Analyst

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TUTORIAL REVIEW

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Coupling isotachophoresis and capillary electrophoresis: a review and comparison of methods

Cite this: DOI: 10.1039/c2an36150g

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We present a comprehensive review and comparison of the methodologies for increasing sensitivity and resolution of capillary electrophoresis (CE) using online transient isotachophoresis (tITP). We categorize the diverse set of coupled tITP and CE (tITP-CE) methods based on their fundamental principles for disrupting isotachophoretic preconcentration and triggering electrophoretic separation. Based on this classification, we discuss important features, advantages, limitations, and optimization principles of various tITP-CE methods. We substantiate our discussion with original simulations, instructive examples, and published experimental results.

Received 17th August 2012
Accepted 21st November 2012

DOI: 10.1039/c2an36150g

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1 Introduction

Capillary electrophoresis (CE) is an analytical separation technique, which enables separation and detection of ionic species based on their differential migration velocities under an applied electric field.[1] CE is widely used in a wide variety of fields including food analysis,[2] molecular biology,[3] and environmental monitoring[4] for the separation and detection of ionic species ranging from small ions to macromolecules and microorganisms.[5] Typical CE experiments involve injection of a sample mixture as a short zone in a capillary or microchannel filled with a background electrolyte (BGE) with a concentration significantly higher than those of analytes, followed by application of an electric field. Thereafter, analyte zones separate based on their differential electrophoretic mobilities. As the analyte zones separate they also disperse (including by molecular diffusion). Measured CE signals are called electropherograms, which quantify the relative heights, widths, and locations of peaks corresponding to separated analyte zones.

The ability of CE to distinguish separated analyte zones is characterized by its resolution, the separation between

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neighboring peaks normalized by their thickness.[6,7] Peaks are said to be well-resolved if the separation between their maxima is significantly larger than their widths. The ability to identify a well-resolved analyte peak over background noise is termed detection sensitivity, and is characterized by the height of the peak relative to the fluctuation magnitude of the background signal.[7] Designing CE experiments often poses a tradeoff between resolution and sensitivity: longer separation times increase peak separation, but at the cost of lower sensitivity due to increased dispersion.[7] In general, CE has very high resolving power, and in many cases higher than that of chromatographic techniques.[8,9] However, in some cases, low sensitivity of CE can limit its applicability, particularly for detection modes other than fluorescence such as ultra-violet (UV) absorbance and various electrical detection methods (*e.g.*, conductivity detection).[10,11]

Several methods have been employed to increase the sensitivity of CE, including use of high-sensitivity detectors, and off-line and on-line sample preconcentration techniques. Direct laser-induced fluorescence (LIF) is the most sensitive method for detection in CE, and has been used for single molecule detection (order 100 aM concentration) with on-line sample preconcentration.[12] However, in practice, many analytes of interest are either non-fluorescent or lack free chemical groups that can be derivatized with fluorophores. Even if fluorescent-labeling of analytes is possible, the inefficiencies involved in derivatization of trace species can result in unwanted artifacts, including modification of analyte mobilities and background signals resulting from unbound fluorophores and their degradation products.[13,14] Detection of many analytes using CE with local conductivity or UV absorbance detection modes is limited to analytes to order 1–100 nM or greater concentrations.[10,11]

Irrespective of the method of detection, detection sensitivity of CE can be maximized by performing sample preconcentration prior to CE. While offline preconcentration techniques such as liquid–liquid extraction and solid-phase extraction are routinely used for sample pretreatment, these methods are often laborious, are time consuming, can require significant experimental skills, and are difficult to automate. In contrast, electrophoresis based online preconcentration techniques, such as field-amplified stacking (FASS),[15,16] dynamic pH junction,[17,18] and isotachophoresis (ITP)[19,20] are well suited for increasing CE sensitivity as they require little or no changes in the existing CE setups, and their coupling with CE can be mostly or completely automated. All electrophoretic sample preconcentration techniques set up specific regions with spatial gradients in electromigration velocities of analyte ions, wherein analytes undergo a net accumulation. Such spatial gradients in electromigration velocity can be generated by creating corresponding gradients in the local electric field or electrophoretic mobility.

ITP is perhaps the most effective and robust among all electrophoretic preconcentration techniques. In ITP, two co-ions of differing mobilities are used to establish steep, self-correcting electric field gradients.[21,22] ITP has been used to achieve more than a million-fold preconcentration[12,23] of sample analytes and the technique can be highly robust to or virtually eliminate interference from sample impurities and non-uniform bulk flow.[24] To perform ITP, a sample mixture is introduced between zones of leading and trailing electrolytes (LE and TE, respectively). LE and TE are chosen such that their co-ions (termed here the LE and TE ions) have respectively higher and lower effective mobilities than those of analytes. When electric field is applied along the channel, analyte ions accumulate and preconcentrate between LE and TE zones. When present in sufficiently large amounts, analytes preconcentrate and separate into contiguous, plateau-like zones with locally uniform concentrations. In contrast, when analytes are present in trace amounts, they focus in the form of peaks within the interface of two neighboring zones. These two modes of operation are termed "plateau-mode" and "peak-mode" ITP.[25,26] Irrespective of the mode of operation, concentration of focused analytes in ITP is typically significantly higher than their initial concentrations. We note, however, that coupling ITP and CE are necessary only when analytes are present in trace quantities, and in such cases analytes typically focus in peak mode ITP.

Besides having the highest preconcentration ability among all electrophoretic preconcentration techniques, ITP has several unique advantages which make it particularly suited for online-preconcentration in CE.[19,20] Firstly, the ultimate preconcentration ability of ITP is independent of the sample conductivity.[24] This is in contrast to other electrophoretic preconcentration techniques in which high conductivity samples can interfere with preconcentration mechanisms and cause significant dispersion of stacked analytes. Further, ITP can be used to selectively focus target analytes while eliminating unwanted impurities. Selective focusing in ITP can be achieved by correctly choosing LE and TE ions whose mobilities bracket only those of the target analytes and not of other contaminant species. Even if excess amounts of impurities are present in the sample, ITP tends to normalize their concentrations to the level of the LE ion concentration. That is, concentrations of trace species and excess impurities tend toward similar concentrations as ITP progresses, decreasing the impurity-to-trace species concentration ratio. Lastly, ITP preconcentration provides an ideal method for sample injection prior to CE as ITP focusing of trace analytes yields a narrow zone of overlapping analyte peaks (typically of order 10–50 μm wide peaks), and this improves separation resolution.[27]

Several methods exist for coupling ITP and CE, and all leverage the fact that disruption of ITP focusing results in electrophoretic separation of analytes. Since coupling of ITP and CE involves an initial, temporary ITP focusing of analytes, these methods are usually referred to as transient ITP-CE (tITP-CE). tITP-CE is the most sensitive electrophoresis separation method, can yield more than a million-fold preconcentration of analytes,[23] and has been used to detect 100 aM initial sample concentrations.[12] Coupling of ITP and electrophoretic separation dates back to at least the 1960s when Ornstein[28] and Davis[29] demonstrated its use for separating proteins from human blood serum. Since then, numerous studies describing new tITP-CE methods, their optimization, and application for analysis of various samples have been published. Coupling ITP and CE has

been a focus of several excellent reviews, including those by Krivankova and Bocek,[30] Timerbaev and Hirokawa,[24] and Mazereeuw *et al.*[31] However existing reviews have either focused on subsets of the ITP and CE coupling methodology and/or on the applications of tITP-CE to certain analyte types. Each tITP-CE method has its own advantages, limitations, applicability to certain analytes, and specific hardware requirements. Therefore we propose a systematic categorization of all these methods here in an effort to help users select an optimal tITP-CE method for their requirements. Moreover, a coherent classification of tITP-CE methods based on their underlying principles may help spark future improvements or entirely new methods for coupling ITP and CE.

We present a systematic framework of categorizing tITP-CE methods based on their fundamental principles of disrupting ITP preconcentration and triggering CE separation. Our classification is illustrated in Fig. 1. Since ITP involves focusing of analyte ions between zones of relatively high mobility LE ions and low mobility TE co-ions, CE can be triggered by either: (1) *disrupting the zone order*, such that LE and TE zones no longer (spatially) sandwich analyte zones, or (2) *changing the mobility order of species*, such that the mobility value of analytes is no longer bound by those of LE and TE ions. Our aim here is to present a comprehensive review of the methodologies used to trigger CE from ITP, and review the important features, advantages, limitations, and optimization principles. Our aim is not to present near-exhaustive listings of tITP-CE applications or related publications (such treatments already exist[24,32]). We instead present only a few instructive examples and specific applications of tITP-CE methods. Further, our discussions are equally applicable to on-chip as well as benchtop electrophoresis setups, and we do not distinguish tITP-CE methods in this regard. We begin by reviewing the relevant principles of ITP and various sample injection techniques. We then present methods for coupling ITP and CE based on disruption of zone order and changing the mobility order of LE, TE, and/or analyte ions. Throughout, we present original model simulations (using our open-source electrophoresis simulation tool, SPRESSO[33,34]) and anecdotes in the form of published experimental results to illustrate specific features of various tITP-CE methods.

2 Sample preconcentration using ITP

The physics, instrumentation, modeling, and applications of ITP are discussed in detail elsewhere[21,35] and will not be reviewed at length here. Instead, in this section, we discuss essential features of ITP that make it well-suited for online preconcentration immediately prior to CE. ITP is an electrophoretic preconcentration technique which leverages discontinuous electrolyte systems to preconcentrate and, in some cases, separates analytes based on their electrophoretic mobility. Fig. 2a shows a schematic of an ITP experiment for preconcentration of two model analytes S1 and S2. A sample mixture consisting of S1 and S2 ions is initially sandwiched between LE and TE zones. LE and TE are chosen such that their co-ions (having valence with same sign as that of analytes) have respectively higher and lower effective mobilities than those of the analytes. Usually, LE and TE have a common counter-ionic

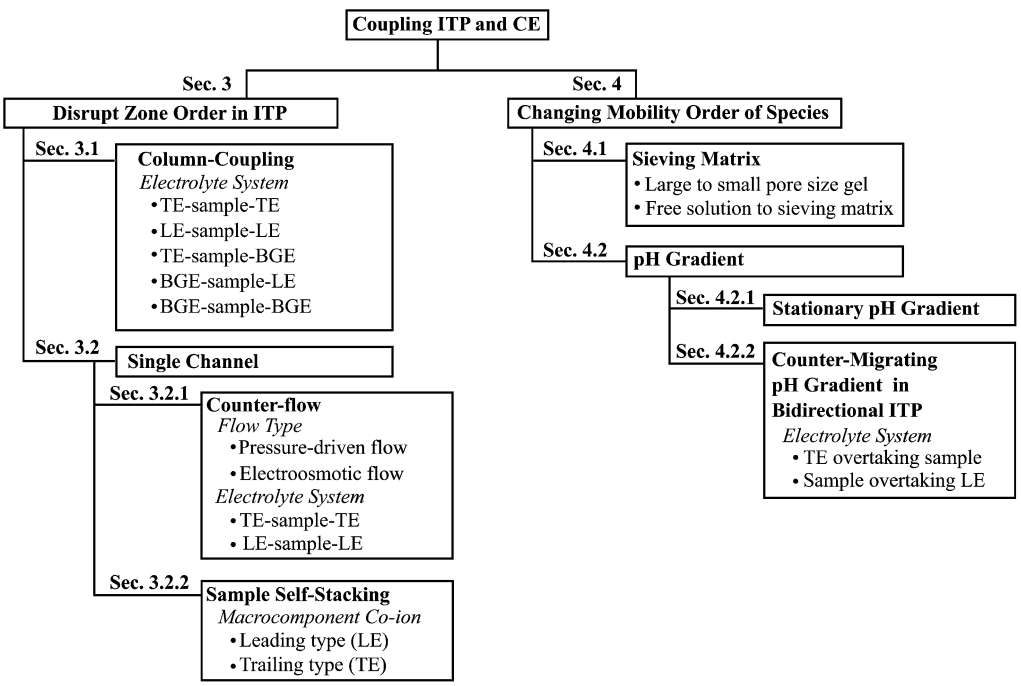

Fig. 1 Classification of methods for coupling ITP and CE. In ITP, analytes focus between zones of high-mobility LE ions and low-mobility TE ions, only if: (i) analyte zones are bracketed by LE zone from front and by TE zone from behind, and (ii) analyte mobilities are such that they fall behind LE ions and race ahead of TE ions. Disruption of one or both of these focusing conditions during ITP triggers electrophoretic separation of analytes. Therefore, we categorize coupled tITP-CE methods into those which involve disruption of ITP focusing of target analytes by either: (i) disrupting the zone order, or (ii) changing the mobility order of LE, TE, and/or analyte ions.

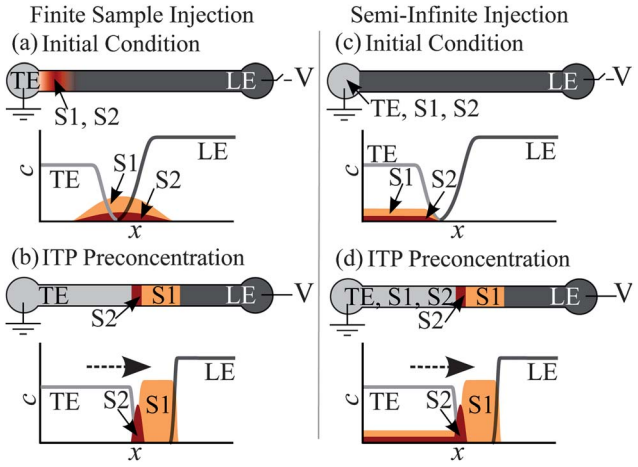

Fig. 2 Schematics illustrating isotachophoretic focusing and separation of two analytes using finite and semi-infinite injection schemes. (a and b) ITP preconcentration and separation using finite sample injection. (a) Initially, a finite zone of an analyte mixture consisting of S1 and S2 ions (electrophoretic mobilities, μ, such that $\mu_{S1} > \mu_{S2}$) is dispensed between LE and TE zones. Analytes are injected by filling the left reservoir with analyte mixture and then applying pressure or electric field. Following analyte injection, the left well is emptied, rinsed, and filled with TE to obtain the necessary initial species distribution for ITP. (b) When an electric field is applied, S1 and S2 focus between LE and TE zones, and analyte zones are ordered according to their mobilities. Here, S1 and S2 focus in "plateau mode" and "peak mode", respectively. These modes are associated with respectively high and low initial analyte concentrations. (c and d) Continuous ITP preconcentration and separation using semi-infinite injection. In the semi-infinite injection scheme, analytes are initially mixed with TE (and/or LE), and are allowed to focus continuously over time. In contrast to finite-injection, analyte zones in semi-infinite injection do not attain steady distributions; instead, the length of plateau zones and concentrations of peak-mode analytes respectively increase continuously over time.

species. When electric field is applied, S1 and S2 migrate towards the electrode with opposite polarity and simultaneously redistribute themselves into contiguous zones, ordered by their electrophoretic mobilities (Fig. 2b); we assume that mobility of S1 is greater than that of S2 everywhere. Redistribution of analyte zones in ITP is accompanied by simultaneous increase in their local concentrations, and this maintains continuity of current while analyte ions displace LE ions.

2.1 Peak-mode *vs.* plateau-mode focusing in ITP

Depending on their initial amounts, analytes in ITP can focus in the so-called plateau-mode or peak-mode.[25,26] For sufficiently high initial analyte concentration and sufficient focusing time, analytes rearrange themselves into "purified" plateau-like zones characterized by locally uniform concentrations and sharp zone boundaries. Maximum plateau zone concentrations are governed by regulating functions such as the Kohlrausch[36] function (for fully ionized species) or more generally the Jovin–Alberty[37,38] function (for monovalent weak electrolyte species). These regulating functions are statements of conservation of charge and continuity of current. Analyte zone concentrations in plateau-mode ITP are usually on the order of, and slightly less than LE ion concentrations. Analyte zones in plateau-mode ITP are separated by sharp zone boundaries which result from a balance of electromigration and diffusive fluxes. These zone boundaries are shock waves in ion-concentrations,[39,40] and their self-sharpening nature prevents them from dispersing further over time.

In contrast to this is the case of peak mode ITP where analytes are present in trace amounts. In peak mode, concentrations of analyte zones do not reach their corresponding plateau values, and instead analytes focus in the form of peaks within the interface of two neighboring zones. Although zone concentrations in peak-mode ITP are considerably smaller than the corresponding plateau concentrations, they are typically significantly larger than initial analyte concentrations. Preconcentration prior to CE can be achieved with either peak mode (*e.g.*, with little or no separation between multiple analytes) or in plateau mode (wherein one or more species has reached plateau mode).

In tITP-CE, analytes initially focus between LE and TE zones. Subsequent disruption of ITP focusing triggers CE, and CE separation can occur within the LE zone, the TE zone, or a new zone (*e.g.*, a newly introduced background electrolyte). LE or TE typically serves as the background electrolyte during the CE step in tITP-CE. If there is a choice between peak or plateau mode ITP for tITP-CE, we advocate the use of peak-mode ITP as a preconcentration mode prior to CE. The reason for this is that disruption of plateau mode ITP often results in fairly extensive dispersion of analytes during the ITP-to-CE transition period. This severe, so-called electromigration dispersion[41] is because plateau-mode ITP analyte zones have concentrations comparable to those of LE and/or TE.[42] Irrespective of this, the ITP preconcentration for increasing sensitivity of CE is most interesting when analytes are present in only trace amounts and so peak-mode ITP is often the *ipso facto* choice. We do note, however, that sample mixtures with trace analyte concentrations can contain relatively large impurity concentrations, and these can focus in plateau-mode during the preconcentration step of tITP-CE. These unwanted plateaus can contribute to significant electromigration dispersion during the CE step and deteriorate separation resolution. As we discuss later in Section 3.1.6, such plateau-mode impurities can be removed immediately prior to CE using, for example, column-coupled channels.

2.2 Finite *vs.* semi-infinite injection

Sample mixtures can be injected in ITP by either finite or semi-infinite injection schemes. In the finite injection scheme, shown in Fig. 2a and b, a finite zone of sample is dispensed between the LE and TE zones, prior to application of electric field. This finite amount of analytes injected into the system results in steady spatial distributions of analytes and steady concentrations. Finite sample injection is often preferred for plateau mode ITP as it yields purified analyte zones during ITP focusing, and these zones can be detected based on specific physicochemical properties of analytes. Also, finite injection is more compatible with complex samples where total contribution of analytes and impurities to sample conductivity may be on the order of the conductivity of LE and TE zones. In this case (and unlike semi-finite injection), finite injection ensures that

analytes are ultimately focused into a steady state distribution which is not a function of their initial concentrations. One drawback of finite injection is that very large injection volumes (*e.g.*, within a channel) may be required to ensure that trace analytes are detectable and/or achieve plateau mode.

One way of increasing the sensitivity of ITP is by loading the sample using semi-infinite injection of analytes, as shown schematically in Fig. 2c and d. In semi-infinite injection, the sample mixture is initially mixed with TE (or LE, or both) and its constituent analytes are allowed to focus continuously over time. In the case of mixing analytes with a TE mixture in a reservoir, this injection scheme is alternatively referred to as electrokinetic injection or electrokinetic supercharging.[43,44] This injection can continue even until the point of detection to maximize sensitivity.[26] In contrast to finite-injection, analyte zones in semi-infinite injection do not attain steady distributions. Instead, the length of plateau zones and concentration of analytes focused in peak-mode increase continuously over time, enabling increase in the amount of analyte focused. The focusing rate in semi-infinite injection can be further enhanced by lowering the ion concentration of the TE as this increases the local electric field and electromigration speed of analytes in the TE reservoir.[26] Although semi-infinite injection increases sample accumulation in ITP, it is typically a good choice only when faced with relatively low conductivity samples. In such cases, TE zone properties (including conductivity, local electric field, and pH) are determined only by TE (not by the sample), and this leads to predictable focusing conditions and ease of interpretation of measurements. Moreover, since the focusing rate in semi-infinite injection depends on the conductivity of the mixture of TE and sample, variability in sample conductivities can affect quantification of target analytes. In this review, we do not classify methods for coupling ITP and CE based on the injection schemes. Unless otherwise stated, all tITP-CE methods reviewed here are equally applicable for finite and semi-infinite injection schemes.

tITP-CE overcomes the limitations of ITP and CE by combining the high sensitivity of ITP with the high resolution of CE. Electrophoretic separation can be triggered from ITP by the sudden disruption of ITP focusing conditions of analytes. Therefore, as we mentioned above, all tITP-CE methods involve either: (1) disruption of zone order, such that analyte zones are no longer led by LE and trailed by TE co-ions or (2) changing the mobility order of analytes, TE, and/or LE ions. In Sections 3 and 4, we present tITP-CE methods based on the disruption of zone order and changes in the mobility order, respectively. Where we believe it is instructive, we present model simulations to illustrate the principles of coupling ITP and CE. Based on both our simulations and published experimental results we discuss the advantages, limitations, and methods for optimization of various tITP-CE techniques.

3 Triggering CE by disrupting zone order in ITP

ITP involves focusing of analytes in narrow zones constrained spatially by zones of high-mobility LE ions in front and low-mobility TE ions behind. Stable ITP zones are a consequence of a discontinuous LE/TE system wherein focused analytes can neither overtake high-mobility LE ions nor lag behind low-mobility TE ions.[21,35] One approach to trigger electrophoretic separation is to disrupt the spatial, front-to-back, LE-sample-TE zone order of ITP such that analytes are no longer led by LE and trailed by TE. The simplest way to do this is to reverse the direction of the applied electric field during ITP, causing complete and immediate reversal of LE, analyte, and TE zone orders. However, this approach does not yield meaningful electrophoretic separations as the associated, prolonged conductivity gradients induced by the long-time mixing of LE and TE co-ions into each other results in excessive electromigration dispersion of analyte zones. This mixing causes conductivity and electric field gradients which act to stretch and mix analyte zones in a way that is highly detrimental to separation.

The highest resolution CE separation following ITP preconcentration is achieved by effecting a process which transitions quickly from the discontinuous LE-sample-TE co-ion system of ITP to a condition approaching a locally uniform BGE for the analytes. A uniform BGE ensures that analyte ions can freely overtake or lag behind co-ions of the BGE without the electromigration-dispersion of analyte zones associated with gradients in electric field caused by BGE conductivity gradients. That is, efficient transition from ITP to CE can be ensured by replacing the LE-sample-TE zone order of ITP by a condition which locally approximates a homogenous BGE and sample mixture. In practice, to simplify the disruption of ITP zone order, either LE or TE is chosen as the BGE for CE. Transitions from ITP to CE are therefore most often achieved by replacing the TE zone with LE, or *vice versa*.[20,30] These tITP-CE electrolyte systems are termed LE-sample-LE and TE-sample-TE systems.[20,30] For example, in the LE-sample-LE approach of tITP-CE, LE ions are introduced behind the TE zone. These newly introduced LE ions overtake the TE zone, migrate into and through the focused analyte zones, while TE ions fall behind. This eventually exposes focused analyte ions to a region whose electric field is dominated by LE ions, forcing the analyte ions to defocus and separate.

The above-mentioned electrolyte systems for coupling ITP and CE can be realized in electrophoresis setups with either: (1) column coupled channels, or (2) a single channel. Although, the principle of coupling ITP and CE by disrupting the LE-sample-TE zone order in ITP is the same for different channel geometries, several important operational differences exist. These include variations in methods of injecting electrolytes, voltage switching, and use of hydrodynamic counter-flow. In Sections 3.1 and 3.2 we present methods for disrupting the zone order in ITP using column coupled and single channel systems, respectively.

3.1 Disrupting zone order in ITP using column-coupled channels

Coupling ITP and CE is most commonly done using a column-coupling arrangement where three channels are arranged in a T-shaped layout.[19,20,27,30,45] Fig. 3 and 4 show a typical column-coupled system consisting of a preconcentration channel

connecting the west reservoir and the junction, and a separation channel connecting the junction and the east reservoir. Analytes first focus in ITP inside the preconcentration channel, and later separate electrophoretically in the separation channel. The preconcentration and separation channels are coupled through a junction with a third channel, which we term the switch channel. The switch channel serves to disrupt the LE-sample-TE zone order of ITP and trigger CE. tITP-CE using column-coupled channels was originally demonstrated by Kaniansky and Marak[19] to overcome the low detection sensitivity of CE and low resolution of ITP. Kaniansky and Marak used tITP-CE based on TE-sample-TE system to detect order

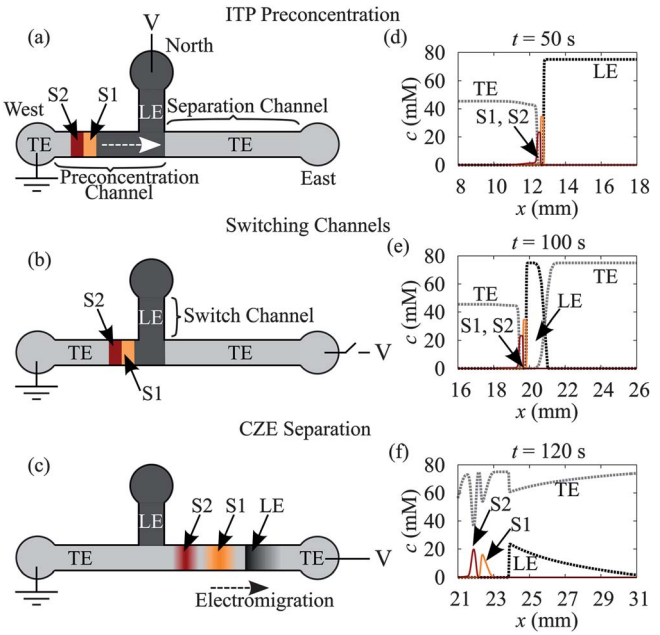

Fig. 3 Schematic and simulation illustrating transient ITP-CE (tITP-CE) based on the TE-sample-TE approach. (a and c) Necessary steps involved in coupling ITP and CE. (a) Initially the separation channel (connecting junction and east reservoir), and the east and west reservoirs are filled with TE, while the remaining channels and the north reservoir are filled with LE. Here we consider the case of finite sample injection, wherein analytes S1 and S2 are initially dispensed between LE and TE zones near the west reservoir. (a) For ITP preconcentration, voltage is applied across the west and the north reservoirs causing S1 and S2 to focus and migrate rightward in the preconcentration channel (connecting west reservoir and junction). (b) When focused analyte zones are about to reach the junction, voltage is switched across the east and the west reservoirs. (c) Thereafter, S1, S2, and residual LE ions separate electrophoretically. (d and f) Simulations illustrating the tITP-CE process based on TE-sample-TE approach. (d) During ITP mode ($t = 50$ s), S1 and S2 focus in narrow zones bracketed by LE and TE zones. (e) At $t = 100$ s electric field is discontinued and most of the LE zone is replaced with TE. A short residual LE zone still remains. This simulation step mimics the channel switching step shown schematically in (b). (f) Subsequent application of the electric field causes analytes and residual LE ions to overtake the TE ions in front of them, and trigger CE. (f) Signal showing three distinct peaks corresponding to S1, S2, and residual LE ions at a later time ($t = 120$ s) during CE. Simulations were performed using our open source code SPRESSO.[33,34] LE was 75 mM hydrochloric acid and 150 mM imidazole; TE was 50 mM tricine and 150 mM imidazole; model analytes S1 and S2 were assumed to be fully ionized with mobilities -20×10^{-9} and -12×10^{-9} m^2 V^{-1} s^{-1}. For simulations we assumed a constant current of 1.4 μA, and a 74 μm wide and 12 μm deep D-shaped, wet-etched channel. We also neglected the effects of ionic strength on species mobility and ionic activity in our calculations.[51]

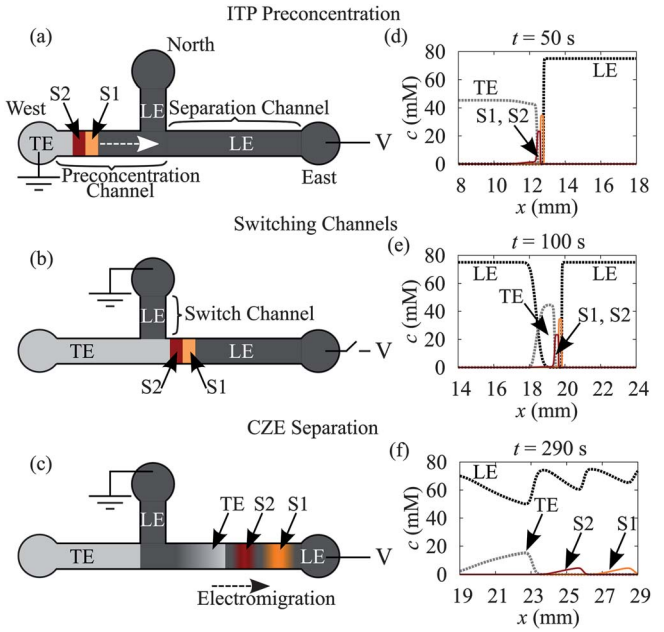

Fig. 4 Schematic and simulation illustrating the tITP-CE process based on the LE-sample-LE approach. (a–c) Necessary steps involved in coupling ITP and CE. (a) Initially all channels and reservoirs are filled with LE, except the west reservoir which is filled with TE. Here we consider the case of finite sample injection, wherein analytes S1 and S2 are initially dispensed between LE and TE zones near the west reservoir. (a) For ITP preconcentration, a voltage is applied across the west and the east reservoirs due to which analytes S1 and S2 focus and migrate rightwards into the preconcentration channel (connecting west reservoir and junction). (b) When focused analyte zones cross the junction, voltage is switched and applied between the north and the east reservoirs. (c) Thereafter, LE ions injected behind the analyte zones overtake S1, S2, and residual TE ions trigger CE separation. (d–f) Simulations illustrating LE-sample-LE based tITP-CE. (d) During ITP mode ($t = 50$ s), S1 and S2 focus in narrow zones bracketed by LE and TE zones. (e) At $t = 100$ s electric field is discontinued and most of the TE zone is replaced with LE. A short TE zone still remains. This simulation step mimics the channel switching step shown schematically in (b). (f) Subsequent application of electric field causes LE ions to overtake the focused analytes and residual TE, and trigger CE. (f) Signal showing three distinct peaks corresponding to S1, S2, and residual TE ions at a later time ($t = 290$ s) during CE separation. Other than the placement of ions, electrolyte chemistry and simulation parameters were the same as those of the simulation shown in Fig. 3.

1 μM concentrations of nitrophenols and amino acids. Since then, numerous studies have been reported on column-coupling based tITP-CE involving new electrolyte systems,[45–47] analysis of complex samples,[24,48] applicability for microfluidic systems,[49,50] and improvements in detection sensitivity.[12,23]

Here, we focus on highlighting the essential steps required for coupling ITP and CE, using all possible electrolyte systems. We also present model simulations and published experimental results to highlight the important features of various tITP-CE electrolyte systems. For all simulations illustrating tITP-CE based on disruption of zone order, we used the chloride ion from 75 mM hydrochloric acid (-79×10^{-9} m^2 V^{-1} s^{-1}, p$K_a = -2$) as the LE ion, 50 mM tricine (-30×10^{-9} m^2 V^{-1} s^{-1}, p$K_a = 8.15$) as the TE ion, and 150 mM imidazole (52×10^{-9} m^2 V^{-1} s^{-1}, p$K_a = 7.15$) as the counter-ion. We used two model analytes S1 and S2, with (fully ionized, absolute) mobilities of -20×10^{-9} and -12×10^{-9} m^2 V^{-1} s^{-1}, respectively. The

analytes S1 and S2 were assumed to be fully ionized throughout the simulations. For clarity of presentation we chose relatively high initial analyte concentrations such that analytes are transitioning from peak mode[25,26] to plateau mode during ITP focusing, and therefore their concentrations only partially overlap. As a supplement to these simulations, we refer interested readers to the fairly comprehensive work of Krivankova *et al.*[20,30,46] on detailed analytical modeling and optimization of these tITP-CE assays.

3.1.1 TE-sample-TE system. The TE-sample-TE approach for coupling ITP and CE in column-coupled channels was first demonstrated by Kaniansky and Marak,[19] and the necessary steps involved in this approach are shown in Fig. 3a–c. Initially, the preconcentration and switch channels, and the north reservoir are filled with LE, while the separation channel and remaining reservoirs are filled with TE. A sample mixture containing S1 and S2 is dispensed at the inlet of the preconcentration channel, between LE and TE zones. To preconcentrate the analytes, a voltage is applied across the west and the north reservoirs (Fig. 3a). Consequently, the analytes focus between LE and TE zones inside the preconcentration channel. Later when the focused analyte zones reach the junction, the voltage is switched across the east and the west reservoirs (Fig. 3b). Consequently, the LE zone is replaced by the TE zone along the direction of the electric field, although a short residual LE zone still remains ahead of focused analytes (Fig. 3c). Thus, the voltage switching step transforms the LE-sample-TE zone order of ITP to TE-sample-TE. Thereafter, analytes S1 and S2, and residual LE ions separate electrophoretically in the separation channel (Fig. 3c) with TE as the background electrolyte. We note that the voltage switching step in Fig. 3b can be performed either manually or it can be programmed using a voltage sequencer. Long delays associated with the manual switching of voltage can lead to increased dispersion of analyte zones. However, this is partially offset by the fact that the residual LE zone in front of the analyte zones can be used to "tighten up" the analyze zones *via* a self-terminating, transient ITP focusing of the analytes immediately after reapplication of the electric field.

Fig. 3d–f show our simulations of the concentration profiles of LE, TE, and analyte ions during preconcentration, transition, and separation steps. Fig. 3d shows S1 and S2 focused in narrow (peak mode) zones between the LE and TE zones during the preconcentration step. Fig. 3e shows the time immediately after replacement of the LE zone in front of the focused analytes with TE. Note the residual LE zone preserving tight analyte peaks. Thereafter, analyte and residual LE ions overtake the TE ions in front of them and start separating electrophoretically. Fig. 3e shows three separated zones corresponding to S1, S2, and residual LE ions.

3.1.2 LE-sample-LE system. The LE-sample-LE approach for coupling ITP and CE is analogous to the TE-sample-TE system, described in Section 3.1.1, except that the TE zone is replaced with LE to initiate CE. Therefore, in the LE-sample-LE system, LE serves as the background electrolyte during CE separation. Early applications of tITP-CE using the LE-sample-LE system in column-coupled channels include works of Stegehuis *et al.*[45] and Krivankova *et al.*[20] Recently Jung *et al.*[12] used this tITP-CE electrolyte system to detect 100 aM fluorescent species in on-chip, column-coupled channels.

Fig. 4a–c show the necessary steps involved in LE-sample-LE based tITP-CE. Initially all channels and reservoirs are filled with LE, except the west reservoir which is filled with TE. The sample mixture consisting of S1 and S2 ions is dispensed between LE and TE zones at the inlet of the preconcentration channel. To preconcentrate the analytes, a voltage is applied across the west and the east reservoirs (Fig. 4a). Consequently, analytes focus into narrow zones bracketed by the LE and TE zones in the preconcentration channel. Later when the focused analyte zones migrate past the junction, the voltage is switched across the north and the east reservoirs (Fig. 4b). This results in sudden injection of LE ions behind the focused analytes, although a short residual TE zone still remains. Thus, the voltage switching step transforms the LE-sample-TE zone order of ITP to LE-sample-LE. Thereafter, LE ions overtake the analyte and residual TE zones, and disrupt ITP focusing. Consequently, S1, S2, and residual TE ions separate into distinct zones in order of their electrophoretic mobilities, as shown in Fig. 4c.

Fig. 4d–f show simulated concentration profiles of LE, TE, and analyte ions during the preconcentration, transition, and separation steps in LE-sample-LE based tITP-CE. Fig. 4d shows analytes focused between LE and TE zones during the preconcentration step. Fig. 4e shows the state at the instant when the TE zone behind the focused analytes is replaced with LE. Note that a short residual TE zone remains behind analyte zones while replacing the TE with the LE zone. Subsequently, LE ions injected behind the analyte zones overtake the analyte and residual TE zones and trigger CE separation. Fig. 4f shows three distinct peaks corresponding to separated S1, S2, and residual TE ions.

3.1.3 BGE-sample-BGE system. The BGE-sample-BGE system was first proposed by Krivankova and Bocek[30] as a generalization of the TE-sample-TE and LE-sample-LE systems for coupling ITP with CE. In the BGE-sample-BGE approach, analytes are first focused between LE and TE during ITP preconcentration. After sufficient focusing of analytes, both LE and TE zones are replaced with a BGE to trigger CE. Depending on their mobilities, analytes either overtake or lag behind the co-ions of BGE, and separate electrophoretically. Unlike the TE-sample-TE and LE-sample-LE systems, where CE is triggered simply by switching the voltage across different reservoirs, coupling ITP and CE with BGE-sample-BGE system requires additional injection steps to replace both LE and TE with BGE.

3.1.4 TE-sample-BGE system. Another variation of the above electrolyte systems for coupling ITP and CE is the TE-sample-BGE system. This approach was first described by Kvasnicka *et al.*[47] and involves replacing the LE zone with BGE to trigger CE. The experimental protocol for this approach is similar to the TE-sample-TE approach shown in Fig. 3a–c, except that in this case the separation channel is filled with BGE instead of TE. Unlike other electrolyte systems, the TE-sample-BGE system allows CE separation of select analytes while retaining ITP focusing for the remaining analytes. As shown by Kvasnicka *et al.*,[47] choosing BGE co-ion with mobility between

those of fastest and slowest analytes in the system yields selective separation of analytes with higher mobility than BGE co-ions. The TE-sample-BGE system is particularly advantageous for analysis of samples containing high-mobility trace analytes and low-mobility macrocomponent species (high-concentration sample constituents), as it can selectively separate trace species without interference from focused macrocomponent species.

3.1.5 Miscellaneous electrolyte systems. A counterpart of the TE-sample-BGE system is the BGE-sample-LE system, wherein the TE zone in ITP is replaced with BGE to trigger selective CE separation of analytes. To our knowledge, BGE-sample-LE based tITP-CE has not yet been demonstrated, but we propose that this approach can be used to selectively defocus and separate *via* CE relatively low-mobility analytes, while retaining high-mobility analytes focused in ITP mode between BGE and LE zones. Given such possible electrolyte combinations for coupling ITP and CE, we propose here a generalized definition of the BGE1-sample-BGE2 system to encompass all possible electrolyte systems. Note that this generalized BGE1-sample-BGE2 definition includes even the TE-sample-LE system which results from the reversal of the electric field during ITP.

3.1.6 Advantages. The column-coupling arrangement has several unique advantages over single channel systems which allow higher detection sensitivity and separation resolution in tITP-CE. Firstly, the cross-sectional areas of the various channel sections can be varied for optimal sample loading and separation.[45] In general, preconcentration and separation channels are chosen with large and small cross-sectional areas, respectively. A large-to-small variation in the channel cross-sectional area is particularly advantageous when the analytes are present in trace quantities and focus in peak-mode ITP. A large cross-sectional area of the preconcentration channel allows for a larger amount of sample loading and accumulation during the ITP preconcentration. Later, after analytes are focused in peak-mode ITP and enter the small cross-sectional area separation channel, their peak concentrations increase due to an increase in local electric field and increase in the amount of analytes focused per unit length in the separation channel.[34,52] Therefore, reduction in cross-sectional area from preconcentration to the separation channel yields higher detection sensitivities during the CE step. Moreover, large surface-to-volume ratios of the narrow separation channel yields improved dissipation of Joule heat, and correspondingly lower dispersion due to temperature gradients.[45]

Another unique feature of the column-coupling arrangement for tITP-CE is the possibility of "cleaning" the sample prior to CE. Complex samples can contain macrocomponent and other contaminating species in concentrations that are much higher than those of trace concentration target analytes. ITP focusing of such samples results in plateau-mode focusing of macrocomponent species and peak-mode focusing of trace analytes. Kaniansky and Marak[19] showed that macrocomponent and contaminant species focused in plateau-mode ITP can be selectively removed prior to CE separation by appropriately switching the electric field and allowing the macrocomponent species to migrate into the switch channel. Such sample cleanup avoids interference by macrocomponent and contaminant species during CE separation and therefore improves separation resolution.

3.1.7 Limitations. The main limitation of column-coupling based tITP-CE is that peak migration times obtained in tITP-CE do not correlate with those obtained in conventional CE experiments. That is, time taken by analyte peaks to reach the detector in tITP-CE is not inversely proportional to the analyte mobilities. Variation in migration times in tITP-CE is a consequence of the effects of the otherwise convenient residual LE or TE zones which enter the separation channel during the transition from ITP to CE (see Fig. 3e and 4e). Residual LE or TE zones result in transient ITP preconcentration during initial moments of the separation step. Consequently, analytes gradually defocus from ITP in the order of their mobility and so initiate CE separation at varying times and distances along the separation channel. This is very much unlike conventional CE where separation begins approximately simultaneously for all analytes.

ITP-to-CE transition in tITP-CE methods based on disruption of the zone order has been studied theoretically and experimentally by Krivankova *et al.*[46] for various electrolyte systems, including the TE-sample-TE, LE-sample-LE, and BGE-sample-BGE systems. Here we summarize the observations of Krivankova *et al.* for the case of the LE-sample-LE system. Krivankova *et al.* showed that in the LE-sample-LE system, the time taken by an analyte (denoted by subscript A) to defocus after injecting LE ions behind the TE zone is given by,

$$t_{\text{ITP-CE}} = \frac{L_T \sigma_T}{j} \frac{(\mu_L - \mu_T)}{(\mu_T - \mu_A)^2}, \quad (1)$$

where, L_T and σ_T, respectively, denote the length and the conductivity of the residual TE zone, and j denotes the current density (assumed to be constant). In eqn (1), μ is the effective electrophoretic mobility of species (assumed to be distinct constants), and subscripts L, T, and A correspond to LE, TE, and analyte ions, respectively. As per eqn (1), an analyte with mobility closer to that of TE ions will take significantly longer time to defocus compared to a higher mobility analyte. Therefore, for the LE-sample-LE system shown in Fig. 4, analyte S2 remains focused for a longer time in ITP compared to analyte S1, as the mobility of former is closer to that of TE ions.

Eqn (1) also shows that the defocusing time depends strongly on the mobilities of LE and TE ions. Therefore, migration times of analyte zones in two tITP-CE experiments with different LE and TE ions will differ, even if all other electrophoresis conditions such as pH and ionic strength are kept constant. This is unlike CE where relative peak migration times do not depend on the mobilities of BGE ions. Variability in the length of residual LE or TE zones can also result in run-to-run variations in peak arrival times in tITP-CE. In the extreme case of long residual LE or TE zones, analytes may not defocus by the time they reach the detector.[46] Unless the aforementioned effects are accounted for, migration times in tITP-CE cannot be used easily for the assignment of analyte peaks. We note that this limitation is inherent to all methods based on disrupting the LE-sample-TE zone order in ITP. In contrast, tITP-CE methods based on the changing order of species mobility (as discussed in Section 4) do not normally exhibit such variability in zone migration times.

3.1.8 Practical considerations. Separation resolution and repeatability of tITP-CE methods based on disruption of zone order can be improved by minimizing the length of residual LE or TE zones during ITP-to-CE transition. Chambers and Santiago[53] experimentally demonstrated the effect of reducing the length of residual TE zone on separation resolution in LE-sample-LE based tITP-CE of DNA fragments. Fig. 5 shows experimental results of Chambers and Santiago, for two different lengths of residual TE zone in the separation channel. Fig. 5a and b show DNA separations for the cases when residual TE zones are 1 and 3 mm long, respectively. Fig. 5c and d show visualization of conductivity gradients using the fluorescent non-focusing tracer technique[53] in separate experiments with similar conditions as those shown in Fig. 5a and b, respectively. Comparison of Fig. 5c and d shows that a smaller residual TE zone yields faster transition to separation as indicated by the increased dispersion of the ITP interface for the case when the TE zone length is a shorter 1 mm (Fig. 5c). Faster transition to CE separation allows DNA fragments to separate for a longer time and yield higher separation resolution, as indicated by comparison of Fig. 5a and b. Lengths of residual LE or TE zones during the separation phase can be minimized by accurately switching the voltage to trigger CE as soon as the focused analytes enter the separation channel. Alternatively, the separation channel can be filled with higher concentration electrolyte compared to the preconcentration channel, so as to establish a step increase in the regulating function.[46] As a result residual LE or TE zones which enter the separation channel readjust to higher concentrations and consequently contract in length.

Faster transition from ITP to CE can also be achieved by appropriately selecting LE and TE ions. As shown by eqn (1), ITP-to-CE transition occurs faster in the LE-sample-LE system if analyte mobilities are much higher than the mobility of TE ions. Therefore, the LE-sample-LE system is better suited for analysis of analytes with mobilities close to that of LE ions. Further, choosing LE as BGE for separation of high-mobility analytes minimizes electromigration dispersion as the difference between mobilities of LE ion and analytes is small. Conversely, the TE-sample-TE system is a better choice for low-mobility analytes as it results in a faster transition to CE, high separation resolution, and lower electromigration dispersion.

3.2 Single channel systems for disrupting zone order in ITP

Electrolyte systems described in Section 3.1 for column-coupling configuration are equally applicable for single channel systems, albeit with a much lower separation resolution. For example, to perform tITP-CE in a single channel using the LE-sample-LE system, analytes are first focused in ITP between the

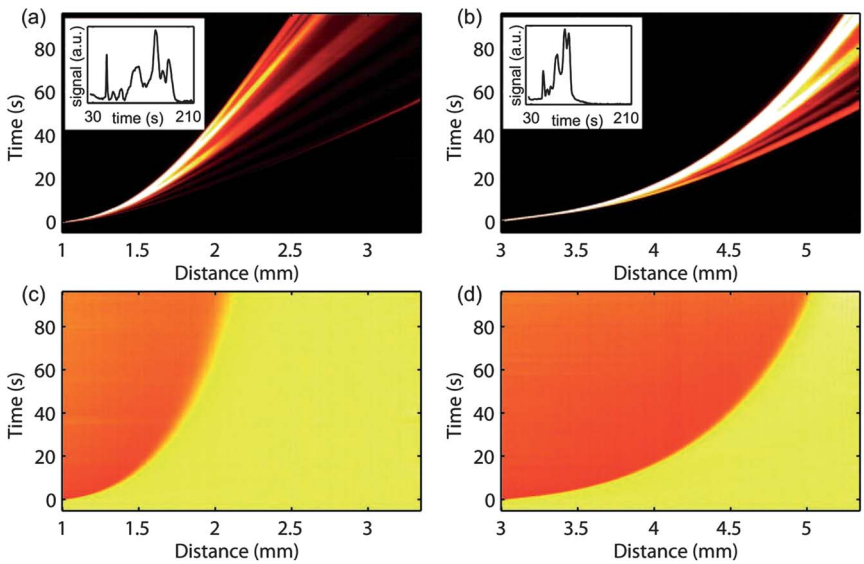

Fig. 5 Experimental results from Chambers and Santiago[53] showing the effect of the residual TE zone on separation resolution in LE-sample-LE based tITP-CE separation of DNA fragments. For these experiments, a 100 bp DNA ladder was first preconcentrated in anionic ITP until focused DNA zones migrated for a predetermined distance past the junction. Subsequently, electric field was switched and applied across different reservoirs to inject LE ions behind the focused DNA fragments. Experiments were performed for different lengths of the residual TE zone formed while switching from ITP to CE. (a) and (b) show spatiotemporal plots (time *vs.* distance) of fluorescent intensity of an intercalating dye (Sybr Green) during ITP preconcentration and CE separation of DNA fragments corresponding to 1 and 3 mm long residual TE zones, respectively. (c) and (d) Show indirect fluorescence based visualization of conductivity gradients in tITP-CE experiments similar to those shown in (a) and (b), respectively. (a and c) When the residual TE zone is relatively short (1 mm), transition to CE is fast and separated DNA peaks are well resolved. (c) Fast transition is also indicated by rapid deceleration and dispersion of the LE/TE interface. (b) For longer (3 mm) residual TE zone, transition to CE is comparatively slow, and hence separation resolution is low compared to the separation shown in (a). (d) Slow transition to separation is corroborated by weaker deceleration and dispersion of the LE/TE interface. Insets of plots (a) and (b) show electropherograms observed at locations 3.5 and 5.5 mm from the channel junction, respectively. LE was 100 mM hydrochloric acid, 200 mM tris, 10× Sybr Green, 100 μM rhodamine-6G (R6G), and 4% PVP (pH 8.1); TE was 38 mM glycine, 3.8 mM barium hydroxide, and 4% PVP (pH 9.0). The DNA ladder was initially dissolved in LE at a concentration of 25 μg mL^{-1}. Indirect fluorescence detection of conductivity gradients was performed by visualizing the fluorescent intensity of R6G dye in various zones. R6G does not focus in ITP, but its concentration adapts to local conductivity gradients allowing indirect and simultaneous quantitation of the electric field in each zone and full-field visualization. The current was held constant at 1 μA. Reprinted with permission from Chambers and Santiago[53] Copyright 2009 American Chemical Society.

LE and TE zones. To inject LE ions behind the TE zone, the electric field is discontinued and the TE reservoir is replaced with LE. Subsequent application of the electric field causes LE ions to overtake the TE and analyte zones and trigger CE separation. Alternatively, LE, TE and the sample can be injected initially into a single channel to achieve the LE-TE-sample-LE zone order. Application of electric field through this electrolyte arrangement results in transient ITP focusing of sample analytes between LE and TE zones. Analyte ions focus in ITP and then separate after LE ions in the rear overtake focused analytes. Irrespective of the injection scheme, single channel systems most often involve relatively longer residual TE zones compared with those in column-coupled systems. As discussed in Sections 3.1.7 and 3.1.8, a longer TE zone in triggering CE with the LE-sample-LE approach yields slow transition to CE and correspondingly low separation resolution. Conversely, reducing the length of TE zone by quickly terminating ITP leads to higher separation resolution, but at the cost of significantly lower net preconcentration. Moreover, run-to-run variability in TE zone lengths due to manual injection of LE ions behind the TE zone in single channel systems can affect the reproducibility of tITP-CE separations. Whereas, column-coupled systems enable a minimization of the length of the residual TE zone by accurate switching of electric field along the various channel sections. Analogous to the LE-sample-LE system, tITP-CE methods based on TE-sample-TE, TE-sample-BGE, and BGE-sample-BGE can be applied in single channel systems, but with significantly lower resolutions compared with the respective methods in column-coupled channels.

Despite the above limitations, tITP-CE in single channel systems have been used to, for example, separate macromolecules,[27,44,54,55] and small ions.[24,43,56] In Sections 3.2.1 and 3.2.2 we review tITP-CE methods which are unique to single channel systems and can yield separation resolutions comparable to those achievable with column-coupled systems.

3.2.1 tITP-CE using hydrodynamic counter-flow. The main drawback of applying LE-sample-LE and TE-sample-TE systems for tITP-CE separations in single channels is slow ITP-to-CE transition and correspondingly low separation resolution. As discussed in Sections 3.1.7 and 3.1.8, minimizing residual TE or LE zones while triggering CE can yield a high separation resolution in tITP-CE. Reinhoud *et al.*[57,58] first demonstrated the use of hydrodynamic counter-flow in tITP-CE to control the length of residual TE and LE zones in LE-sample-LE and TE-sample-TE systems, respectively. In counter-flow based tITP-CE methods, bulk flow is used to oppose the migration of ITP zones during the preconcentration step. By precisely controlling the flow rate, focused analyte zones can be positioned accurately to virtually any desired location along the channel, immediately prior to triggering CE. Hydrodynamic counter-flow during ITP preconcentration in tITP-CE can be applied either by external pressure[57–60] or using electroosmotic flow.[61,62]

3.2.1.1 External pressure-driven counter-flow. Reinhoud *et al.*[57,58] used external pressure-driven flow to control lengths of LE and TE zones during the ITP step of tITP-CE. Although, Reinhoud *et al.* described application of both TE-sample-TE and LE-sample-LE systems, here we limit our discussion to the LE-sample-LE system. Application of the TE-sample-TE system in counter-flow based tITP-CE follows analogously.

Fig. 6 shows the necessary steps required for coupling counter-flow ITP and CE in a single, straight channel using the LE-sample-LE approach. Initially, the channel and the right reservoir are filled with LE and the left reservoir is filled with TE. Here we consider the case of semi-infinite sample injection, wherein analytes S1 and S2 are initially mixed with TE (Fig. 6a). When electric field is applied, S1 and S2 accumulate continuously at the LE/TE interface. Simultaneously, an external pressure is applied across the channel to flow bulk fluid in a direction opposite to the electromigration of analytes. Electric field and counter-flow are adjusted to bring analyte zones to a rest near the TE reservoir, as shown in Fig. 6b. After a sufficient amount of analytes have focused, the electric field is discontinued and TE in the left reservoir is replaced with LE (Fig. 6c). Subsequent application of electric field causes LE ions to overtake the analyte and TE zones and trigger CE separation, as shown in Fig. 6d. Note that in the above discussion we assumed that electroosmotic flow (EOF) is suppressed and negligible compared to electromigration velocities. However, in cases where EOF is significantly large and opposite to the electromigration of analytes, reversal of the electric field during the separation may be necessary to ensure that the analytes migrate towards the opposite end of the channel.[57,58,60]

Advantages. Irrespective of the injection scheme, finite or semi-infinite, counter-flow ITP preconcentration prior to CE separation allows significantly large sample loading and correspondingly high detection sensitivities. Reinhoud *et al.*[57,58] used finite sample injection by completely filling the separation channel with the sample mixture, and dispensing LE and TE into the end-channel reservoirs. During ITP preconcentration, Reinhoud *et al.* used hydrodynamic counter-flow to remove the TE zone and position the focused analyte zones near the TE well. Semi-infinite injection is even better suited for counter-flow ITP preconcentration, as analytes can be focused for an indefinite time period by counterbalancing electromigration and bulk-flow and holding analyte zones stationary. Urbanek *et al.*[59] leveraged this feature of semi-infinite injection and counter-flow ITP to focus analytes over 1 h prior to CE separation, and demonstrated a detection of order 1–10 ppb (~10 nM) unlabelled impurities in butylmethylimidazolium-based ionic liquids. Recently, Davis *et al.*[60] used a similar approach and demonstrated separation and detection of six fluorescently labeled amino acids with detection limits as low as 200 fM, and a 11 min assay time.

Aside from increasing detection sensitivity, stationary ITP focusing in counter-flow ITP can also be used for complete removal of sample macrocomponent and contaminant species prior to CE separation. Sample clean-up can be achieved by choosing LE and TE mobilities which selectively bracket mobilities of target analytes, but not of sample macrocomponent and contaminant species. As a result, macrocomponent species and impurities which do not focus between LE and TE zones either migrate to the LE reservoir if they have higher mobility than LE ions or they are driven to the TE reservoir by the counter-flow.[57,58] Significantly long focusing

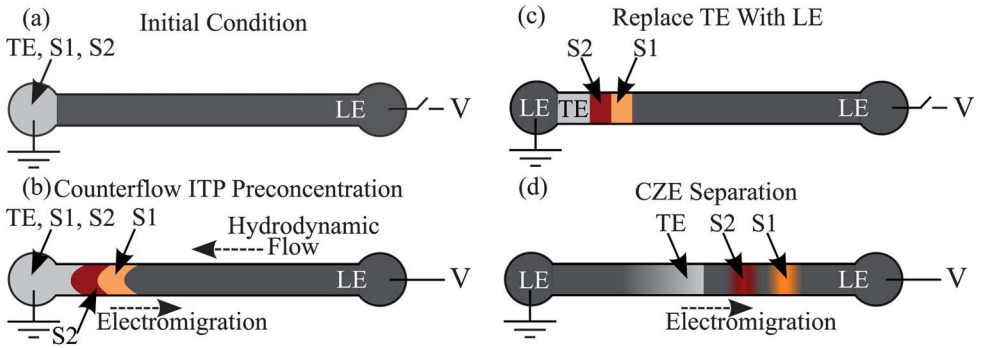

Fig. 6 Protocol for performing tITP-CE in a single, straight channel using hydrodynamic counter-flow. Shown here is the LE-sample-LE system for coupling ITP and CE. (a) Initially the channel and the right reservoir are filled with LE and the left reservoir is filled with a mixture of TE and analytes (S1 and S2). (b) When the electric field is applied, S1 and S2 focus and accumulate continuously between LE and TE zones. During analyte focusing, a hydrodynamic counter-flow is applied to bring the focused analyte zones to a rest near the TE reservoir. Counter-flow, in principle, allows sample focusing for indefinite time and also provides better control of the length of TE zone inside the channel. The latter feature of counter-flow based tITP-CE helps improve separation resolution by minimizing the length of TE zone while triggering CE. (c) After sufficient amount of analytes have focused, the electric field and hydrodynamic counter-flow are simultaneously discontinued, and TE in the left well is replaced with LE. (d) Subsequent application of electric field causes LE ions to overtake zones of the residual TE zone, S1, and S2, and thereby trigger electrophoretic separation.

times in counter-flow ITP allow complete removal of non-focusing species from the sample mixture prior to triggering CE.

Limitations and practical considerations. Counter-flow based tITP-CE requires precise control over external pressure to reproducibly achieve similar lengths of residual LE or TE zones while triggering CE. Otherwise, associated variability in ITP-to-CE transition times can lead to variations in the amount of sample loaded and in peak migration times. As noted by Reinhoud *et al.*[57,58] variability in location of the analyte zones is even more severe when significant EOF is present, as EOF mobility can vary over the course of counter-flow tITP-CE experiments. Therefore, active and precise control over external pressure is necessary during counter-flow based tITP-CE experiments. Also, for better repeatability, counter-flow based tITP-CE experiments should be performed in channels with properly conditioned surfaces and using well-buffered electrolyte solutions to ensure consistent EOF during different runs.

3.2.1.2 Electroosmotic flow. Breadmore[61] presented an alternate method for achieving stationary analyte zones in tITP-CE by counter-balancing electromigration of analyte zones with EOF. Breadmore demonstrated stability of analyte zones for over 1 h during ITP preconcentration with semi-infinite injection of analytes. Subsequent CE separations triggered by injecting LE ions behind the TE zone showed 10 000-fold higher detection sensitivity compared to CE experiments with finite sample injection. In a later study, Breadmore and Quirino[62] presented a further 10-fold improvement in the analyte focusing rate in EOF based counter-flow ITP by using relatively low conductivity TE for semi-infinite injection of analytes. Leveraging high focusing rates due to low-conductivity TE and long focusing times, Breadmore and Quirino demonstrated tITP-CE separation of inorganic ions with order 100 pM detection sensitivity using UV detection.

Advantages, limitations and practical considerations. An advantage of using EOF over pressure-driven flow in counter-flow based tITP-CE is that the former approach does not require an external pressure controller. This advantage, however, comes at the significant cost of having less control over the length of the residual TE zone in LE-sample-LE based tITP-CE. Assuming a uniform EOF mobility of channel surface, the length of the TE zone in EOF based counter-flow ITP is given by[63]

$$\frac{L_{TE}}{L} = \left(1 + \frac{\mu_{LE}}{\mu_{EOF}}\right)\left(1 - \frac{\mu_{LE}}{\mu_{TE}}\right)^{-1}, \quad (2)$$

where the ratio L_{TE}/L denotes the fraction of the total channel length (L) occupied by the TE zone. In eqn (2), μ_{LE} and μ_{TE} respectively denote the mobilities of LE and TE ions, and μ_{EOF} denotes the EOF mobility of channel surface. Note that eqn (2) is valid only when μ_{EOF} and μ_{LE} have opposite signs and $|\mu_{TE}| < |\mu_{EOF}| < |\mu_{LE}|$. That is, stationary analyte zones result only when EOF acts against electromigration of analyte ions and EOF mobility magnitude is bracketed by those of LE and TE ions. Therefore, EOF based counter-flow tITP-CE is applicable only if target analytes and channel surface are like-charged. For example, glass channels with negatively charged surface cannot be used for EOF based counter-flow tITP-CE separation of cations, as EOF acts along the direction of electromigration of cationic analytes.

Eqn (2) also suggests that a given electrolyte chemistry can yield at most one location in the channel where analyte zones can come to rest. Further, location of stationary analyte zones and the length of the TE zone in EOF based counter-flow ITP depend strongly on the mobilities of LE and TE ions. Since the length of the TE zone determines ITP-to-CE transition time, different electrolyte systems can yield different separation resolutions. This is not the case for pressure-driven counter-flow based tITP-CE as the length of the TE zone and therefore separation resolution can be tuned by simply varying the external pressure.

3.2.2 Sample self-stacking in CE. Until now, we presented tITP-CE methods where both LE and TE ions were included to effect ITP preconcentration. However, in some cases the samples themselves contain macrocomponent species which can serve as LE or TE ions. This is usually the case for many

biological and environmental samples, such as urine, serum, and sea water, which contain large concentrations of high-mobility sodium and chloride ions. For such samples, sodium and chloride ions can be used as LE ions for cationic and anionic ITP, respectively, prior to CE. Since preconcentration of analytes is induced by the components of the sample itself, tITP preconcentration using sample mixtures containing an inherent LE or TE co-ion is termed sample self-stacking.[64] We note that if such macrocomponent species are not leveraged for sample self-stacking in CE, they will instead interfere with CE separation and cause electromigration dispersion of analyte zones.

Sample self-stacking in CE was first reported by Verheggen *et al.*[65] and Schoots *et al.*[66] for separation of uric and hippuric acid in blood serum. Schoots *et al.* showed that addition of chloride to diluted serum samples in CE experiments resulted in sharper peaks, indicating transient ITP preconcentration. Later, Gebauer *et al.*[64] presented an analytical model and simulations of sample self-stacking in CE and showed that its preconcentration ability is equivalent to that of FASS-CE of similar samples but without macrocomponent ions; FASS-CE applies only to low-conductivity samples. Applications of sample self-stacking in CE have been reviewed in detail by Timerbaev and Hirokawa.[24] Here, we review important steps required to perform and optimize sample self-stacking in CE for cases where sample macrocomponent species serve as LE and TE ions for transient ITP.

3.2.2.1 Macrocomponent ion of leading type. Sample self-stacking experiments resemble usual single channel CE experiments, except that the BGE is chosen appropriately to ensure transient ITP preconcentration. When the sample contains high-mobility macrocomponent species which acts as the LE ion during transient ITP preconcentration, TE is chosen as the BGE for tITP-CE (*i.e.*, of the TE-sample-TE type). Fig. 7 shows a schematic of sample self-stacking based tITP-CE analysis of a sample containing macrocomponent species of leading type (labeled as LE). Initially, a single, straight channel and both reservoirs are filled with TE, and the sample mixture consisting of high-mobility macrocomponent species (LE) and two analytes (S1 and S2) is dispensed at the left end of the channel (Fig. 7a). When the electric field is applied, analytes S1 and S2 stack behind the LE zone, as shown in Fig. 7b. Simultaneously, the leading interface of the LE zone starts dispersing into the TE zone ahead of it as the mobility of LE ions is higher than that of TE ions, whereas the trailing interface of LE zone remains sharp due to the isotachophoretic effect. At later times, analyte zones transition from ITP to CE and separate based on their electrophoretic mobilities, as shown in Fig. 7c. We note that the case shown in Fig. 7 is just a special case of the TE-sample-TE system (discussed in Section 3.1.1) in a single channel system, where the sample is initially mixed with LE.

3.2.2.2 Macrocomponent ions of the trailing type. tITP-CE separation of analytes from the sample mixture containing low-mobility macrocomponent ions is analogous to the case of high-mobility macrocomponent ions, except that in this case macrocomponent species act as the endogenous TE ions. Therefore, LE is chosen as the BGE to ensure transient ITP focusing of analytes prior to CE. This approach is similar in principle to the LE-sample-LE approach discussed in Section 3.1.2.

3.2.2.3 Practical considerations. Sample self-stacking in CE is similar in principle to the TE-sample-TE and LE-sample-LE systems described in Sections 3.1.1 and 3.1.2, respectively. Besides usual considerations involved in using these tITP-CE electrolyte systems, as discussed in Sections 3.1.8, their use in sample self-stacking-based tITP-CE poses an additional tradeoff between detection sensitivity and resolution. Since target analytes are initially mixed with LE or TE, higher sample loading is accompanied by longer LE or TE zones, which in turn results in longer ITP-to-CE transition times and lower separation resolution (as discussed in Sections 3.1.7 and 3.1.8). This is in contrast to column-coupling and counter-flow based tITP-CE techniques where sample accumulation during ITP can be increased (by cross-sectional area reduction or counter-flow) without affecting the length of residual LE or TE zones during ITP-to-CE transition.

Detection sensitivity and separation resolution in sample self-stacking based tITP-CE can also be affected by the presence of additional co-ionic macrocomponent species which interfere with sample stacking. Gebauer *et al.*[67] presented a theoretical and experimental study on the effect of an additional co-ionic macrocomponent species (with lower mobility than analytes) on leading-type sample-stacking (*i.e.*, the TE-sample-TE type). Gebauer *et al.* showed that leading-type sample stacking of a particular analyte occurs only when the relative concentration of leading-type macrocomponent species (LE) exceeds a critical value given by,

$$\frac{c_{LE}}{c_M} > \frac{\mu_M - \mu_C}{\mu_{LE} - \mu_C}\frac{\mu_{LE} - \mu_A}{\mu_A - \mu_M}. \quad (3)$$

where subscripts M and A denote the additional, low-mobility macrocomponent species and the target analyte, respectively. If the inequality in eqn (3) is not obeyed by the sample mixture, analytes do not focus in ITP. Instead, analyte zones disperse due to electromigration-dispersion induced by high-conductivity of the sample mixture. Note that the inequality in eqn (3) holds only for electrolyte systems consisting of fully ionized species. Gebauer *et al.*[67] also presented an analogous result for electrolyte system with weak monovalent species.

In practice, diluting a sample containing leading-type macrocomponent species (LE) with a trailing-type BGE co-ion will also yield lower sample-stacking due to the disruptive effect of slow BGE co-ions. For such cases, eqn (3) can still be used to estimate the required concentration of LE ion in the sample, except that now the BGE co-ion is the additional macrocomponent species (M). Fig. 7d shows results from sample self-stacking experiments of Gebauer *et al.*[67] for five different ratios of LE-to-BGE ion-concentrations in the sample mixture. Electropherograms in Fig. 7d show that detection sensitivity (peak heights) and separation resolution (peak separation) increase for higher ratios of LE-to-BGE ion-concentrations (denoted by a) in the initial sample. Also, when the concentration of LE ions in the sample mixture is significantly smaller than the concentration of trailing-type BGE co-ions, no sample stacking is observed. Instead, CE separations yield highly dispersed analyte zones.

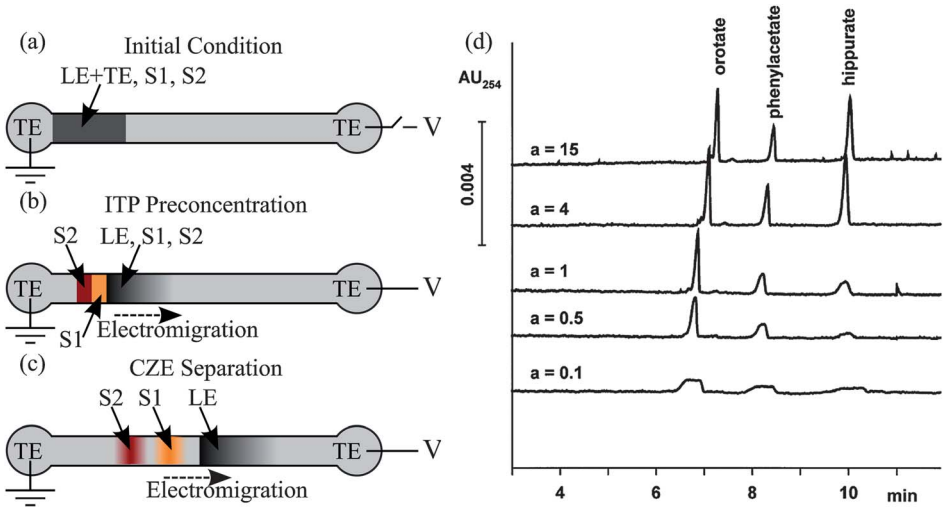

Fig. 7 Schematics (a–c) and experimental results from Gebauer *et al.*[67] (d) Illustrating sample self-stacking in CE. Shown here is a case for sample mixture containing a high-mobility, leading-type of macrocomponent species (LE). Accordingly, TE is chosen as the BGE for CE to ensure sample stacking between LE and TE zones. (a) Initially, the separation channel and both reservoirs are filled with TE. Sample mixture consisting of S1, S2, and LE ions is dispensed at the left end of the channel. Here, we consider a general case when initial sample zone also contains TE ions. (b) When electric field is applied, analytes (S1 and S2) focus at the trailing end of LE zone. Simultaneously, the leading interface of LE zone disperses into the TE zone analogous to the TE-sample-TE system shown in Fig. 3. (c) At a later time, LE zone disperses completely in the background electrolyte and analyte zones separate electrophoretically. (d) Electropherograms from Gebauer *et al.*[67] illustrating the effect of TE ion concentration in the sample mixture on detection sensitivity. Peak signal increases with increase in ratio of LE to TE ion-concentration ($a = c_{LE}/c_{TE}$) in the sample mixture. Analytes were 10 μM each of orotate, phenylacetate, and hippurate ions; LE ion was lactate, and TE ion was ACES. Background electrolyte (TE in the current case) consisted of 15 mM ACES, histidine, and 0.005% hydroxypropyl cellulose (HPC) at pH 6.5. The ratio of LE to TE ion concentration was varied in the sample mixture by mixing 12 mM lactate and 0.8 mM ACES ($a = 15$); 8 mM lactate and 2 mM ACES ($a = 4$); 5 mM lactate and 5 mM ACES ($a = 1$); 3 mM lactate and 6 mM ACES ($a = 0.5$); and 1 mM lactate and 10 mM ACES ($a = 0.1$). Experiments were performed in a 100 μm diameter and 37 cm long, uncoated capillary with a detection length of 30 cm. Voltage was held constant at 10 kV and separated analyte peaks were detected using UV absorbance at 254 nm wavelength. (d) Reprinted from Gebauer *et al.*[67] Copyright 2000, WILEY-VCH Verlag GmbH.

4 Triggering CE from ITP by changing mobility order of species

In Section 3 we presented various methods for coupling ITP and CE based on disruption of zone order of LE, TE, and analyte ions in ITP. Here, we describe an alternate approach to couple ITP and CE by changing the mobility order of analytes, LE, and/or TE ions in ITP. In ITP, analytes focus only if their mobilities are bracketed by those of high-mobility LE ions and low mobility TE ions. More exactly (for weak electrolytes) ITP analytes focus if their effective mobilities in the LE and TE zones are such that analytes race ahead of TE co-ions and fall behind LE co-ions (and if TE and LE ions themselves have such self-restoration).[68] Violating these conditions by changing electrophoretic mobility(ies) causes analytes to fall out of ITP mode and separate based on their electrophoretic mobilities. There are two primary ways of changing the order of species mobilities in ITP to trigger CE. The first method is mostly applicable to tITP-CE of macromolecular analytes, such as nucleic acids and proteins, whose mobilities are strong functions of the local concentration of a sieving medium. In this approach, a sharp, spatial gradient in sieving matrix concentration is employed to shift the mobility of focused macromolecules below the mobility of TE ions, thereby initiating CE. The second method of changing the mobility order of species primarily leverages the effects of spatial pH gradients on weak electrolyte ions to change the relative magnitudes of effective mobilities of analyte, TE, and/or LE ions.

Besides using gradients in sieving medium and local pH, electrophoretic mobility can also be affected by (1) variations in the local ionic strength of solution,[51] (2) presence of a micellar phase,[69] (3) complex forming equlibria (*e.g.*, using cylcodextrin[70] and crown ethers[71]), (4) using zones of different solvents,[72] and (5) temperature gradients in time and/or space.[73] The latter modifications of electrophoretic mobilities have been used to vary relative mobilities of species in ITP and CE to increase separation resolution.[51,70,72] However, we know of no studies where ionic strength gradients, micellar phase, complex forming equilibria, variations in solvent, and/or temperature gradients have been used to trigger CE from ITP. Instead, some of these effects are more easily and generally employed as a candidate replacement for ITP as a preconcentration method. For example, differences in ionic strength and solvents can be used to generate local conductivity gradients and thereby stack sample species using the FASS mechanism.[74,75] Partitioning of analytes between the electrolyte buffer and micellar phase is often used to preconcentrate analytes in the sweeping mode of micellar electrokinetic chromatography (MEKC).[69] Further, mobility and conductivity gradients associated with temperature gradients can be used to focus analytes in the temperature gradient focusing technique.[73] We focus here on methods for coupling only ITP and CE, and refer the interested readers to reviews by Breadmore *et al.*,[76–78] which discuss coupling of CE with a wide variety of preconcentration methods, including FASS, MEKC sweeping, and ITP.

Below we discuss methods for coupling ITP and CE based on changing the mobility order of species in ITP using spatial gradients in sieving matrix and pH. These methods are equally applicable for single and column-coupled channels; and we do not categorize them based on the type of channel geometry or injection method.

4.1 Changing mobility order using spatial gradient in sieving matrix concentration

Coupling ITP and electrophoretic separation by changing the mobility order was first demonstrated by Ornstein[28] and Davis[29] for preconcentration and separation of proteins in polyacrylamide gels. The method of Ornstein and Davis is based on performing ITP along a spatial gradient in the pore size (large to small) of polyacrylamide gel. Initially, proteins focus between LE and TE zones inside the large pore size gel. At a later time, when the focused protein zones reach and enter the small pore size region, their mobilities decrease below the mobility of TE ions. Consequently, previously focused proteins lag behind the LE/TE interface and separate in the order of their electrophoretic mobilities. Later, van der Schans *et al.*[79] presented a related technique for tITP-CE separation of DNA fragments based on their differential mobility in free solution and a linear polyacrylamide based sieving matrix. We note that neither Ornstein nor Davis referred to this tITP-electrophoresis method as preconcentration with "isotachophoresis" as the term isotachophoresis was coined later in 1970 by Haglund.[80] However, the approach of Ornstein and Davis is clearly ITP, and it is extensively applied and cited in the proteomics community (*e.g.*, these two papers currently have a combined 27 000 citations according to ISI Web of Knowledge).

Fig. 8 shows a schematic of the tITP-CE method of van der Schans *et al.*[79] Initially, the channel and the right reservoir are filled with LE, while the left reservoir is filled with TE as shown in Fig. 8a. The right half of the channel is filled with a sieving matrix which is required to initiate electrophoretic separation. Analytes S1 and S2 are dispensed between LE and TE zones at the left end of the channel. When electric field is applied, analytes S1 and S2 focus between LE and TE zones in free solution and migrate rightward (Fig. 8b). At a later time, focused analyte zones enter the sieving matrix, which lowers analyte mobilities below the mobility of TE ions. Thereafter, analyte zones lag behind the LE/TE interface and separate in the TE zone, as shown in Fig. 8c. We note that instead of transitioning from free solution to a sieving matrix, CE separation can also be triggered by having a spatial gradient in the sieving matrix concentration, as demonstrated by Ornstein[28] and Davis.[29]

Fig. 8d and e show simulated concentration profiles corresponding to ITP and CE steps shown schematically in Fig. 8a and c, respectively. For these simulations, we assumed that analytes (S1 and S2) are fully ionized and have equal free-solution mobilities of -20×10^{-9} m^2 V^{-1} s^{-1}. We also assumed that the sieving matrix reduces the mobility of S1 and S2 by factors of 2.5 and 5.0, respectively, but does not appreciably affect the mobilities of LE, TE, and counter-ion species. In this regard, our simulation mimics samples S1 and S2 as DNA fragments of two lengths (since the mobilities of DNA fragments are similar in free-solution and strongly dependent on their lengths in a sieving medium[81]). Fig. 8d shows S1 and S2 focused together in a narrow zones bracketed by LE and TE zones, prior to entering the sieving matrix. Fig. 8e shows separated zones of S1 and S2 after they enter the sieving matrix. Note that the LE/TE interface remains intact during the separation phase as the sieving matrix does not appreciably affect the mobilities of LE and TE ions.

4.1.1 Advantages. Compared to conventional CE, tITP-CE based on mobility changes triggered by gradients in a sieving matrix yields much higher sensitivity and highly reproducible peak migration times, and these improvements are largely independent of sample conductivity. For mobility changes effected by gradients in the sieving matrix, CE is triggered only long after initial ITP transients have subsided. Further, CE is triggered by a relatively quick disruption of ITP conditions for all macromolecular analytes. This makes the CE separation independent of the transients of the preconcentration step. We note that absolute peak migration times in CE can be affected by biases resulting from run-to-run variabilities of the initial ITP transients. However, these biases in peak migration times can be eliminated by measuring peak migration times relative to the arrival time (at the detector) of the intact LE/TE interface. This feature of sieving matrix induced tITP-CE is particularly important for base-pair assignments in DNA separations, as conductivity of actual DNA sample may vary vastly from that of the reference DNA ladder. Van der Schans *et al.*[79] demonstrated this by comparing peak arrival times of a 118 bp double stranded DNA (ds-DNA) fragment in DNA separations of a 118 bp PCR product and a reference DNA ladder, using both CE and sieving matrix induced tITP-CE. Fig. 9 shows experimental results of van der Schans *et al.* Fig. 9a shows electropherograms measured during CE separation of the ΦX174/*Hae*III ds-DNA ladder (reference) and a 118 bp PCR product. Both electropherograms were obtained using the same electrophoresis conditions, including same BGE, sieving medium (3% polyacrylamide in 50 mM tris–borate, pH 8.3), and sample injection lengths. However, differences in the sample composition of the DNA ladder and PCR products result in variations in migration times for the 118 bp peak. Fig. 9b shows corresponding electropherograms measured using ITP-to-CE transition triggered by a gradient in the sieving matrix. Peak migration times shown here are relative to the migration time of the ITP interface, which can be detected due to focused impurities during the CE step. Unlike separations using CE, electropherograms measured in tITP-CE separations of the PCR product and the reference DNA ladder show similar arrival times of the 118 bp fragment peak, allowing accurate base-pair assignment.

In addition to providing more reproducible separations, tITP-CE induced by sieving matrix gradients also seems to yield significantly higher separation resolution than all tITP-CE methods based on disruption of zone order in ITP (discussed in Section 3). We attribute this to the fact that in the sieving matrix gradient method, analytes defocus and separate as soon as they enter the region of high-concentration sieving matrix. This rapid ITP-to-CE transition induced by the sieving matrix results

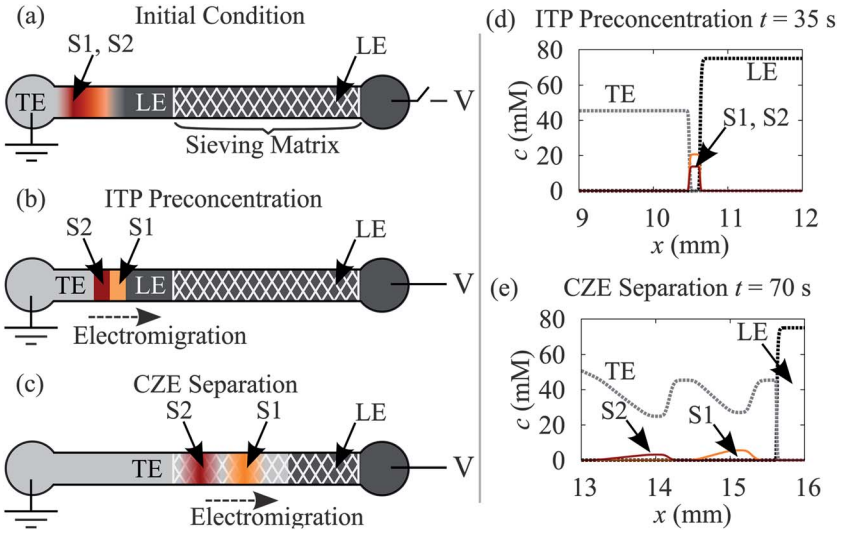

Fig. 8 Schematics (a–c) and simulation (d–e) tITP-CE based on changing analyte mobilities through gradients in sieving matrix. (a) Here, the channel and the right reservoir are first filled with LE, and the left reservoir is filled with TE. Analytes are dispensed between LE and TE zones at the left end of the channel. Also, a portion of the channel towards the right contains the sieving matrix for triggering CE. (b) When the electric field is applied, analytes S1 and S2 focus between LE and TE zones in free solution and migrate rightwards. (c) Later, when focused analyte zones enter the sieving matrix, analyte mobilities decrease below the mobility of TE ions. As a result, analyte zones lag behind the LE/TE interface and separate in the TE zone. (d–e) Simulation illustrating ITP preconcentration in free solution, followed by CE separation in the sieving matrix. For these simulations we used two fully ionized, model analytes (S1 and S2) having equal free solution mobilities of -20×10^{-9} m^2 V^{-1} s^{-1}. We also assumed that the sieving matrix reduces the mobility of S1 and S2 by factors of 2.5 and 5.0, respectively. (c) Since free solution mobilities of analytes are equal, S1 and S2 focus together in a single, narrow ITP zone. (d) When the focused analyte zones enter the sieving matrix (at $x = 12$ mm), their mobilities are reduced to such an extent that S1 and S2 lag behind the LE/TE interface. This triggers CE separation of S1 and S2 in the TE zone. LE, TE, and other simulation parameters were same as those in the simulation shown in Fig. 3.

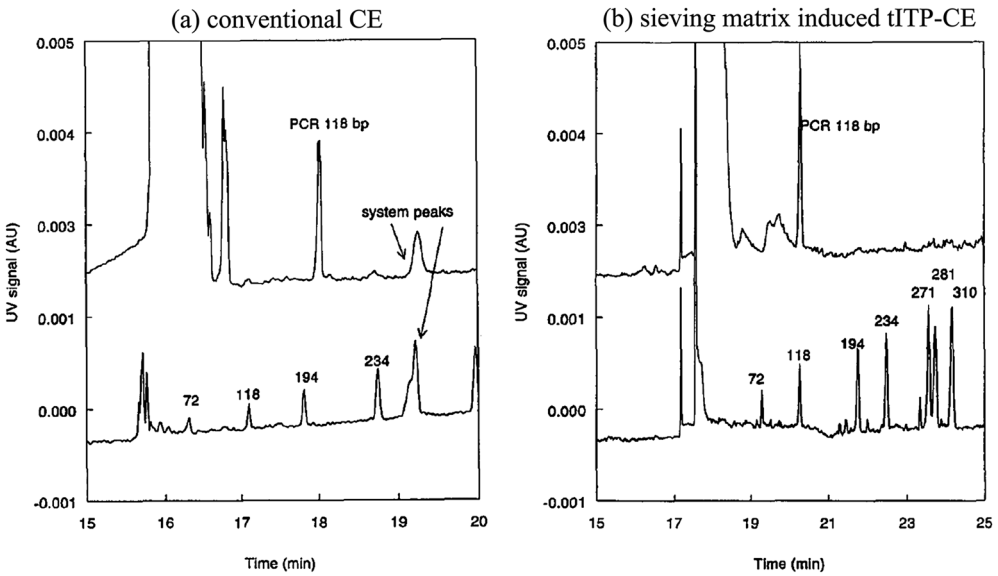

Fig. 9 Results from van der Schans *et al.*[79] showing comparison of base-pair assignment of 118 bp PCR product in conventional CE and tITP-CE based on mobility changes induced by sieving matrix. (a) Electropherograms showing separation of the ΦX174/*Hae*III ds-DNA ladder (bottom) and a 118 bp PCR product (top), both using conventional CE. Both electropherograms were obtained using the same electrophoresis conditions, including same BGE, sieving medium (3% polyacrylamide in 50 mM tris–borate, pH 8.3), and sample injection lengths. However, differences in sample composition and conductivity of the DNA ladder and PCR products yield different migration times for the 118 bp peak. (b) Separation of the ΦX174/*Hae*III ds-DNA ladder (bottom) and a 118 bp PCR product (top) using tITP-CE induced by gradients in the sieving matrix. Sharp peaks around $t = 17$ s correspond to impurities which remain focused at the LE/TE interface during separation of DNA fragments. Comparison of peak migration times of DNA fragments relative to the migration time of ITP interface yields accurate base-pair assignment. tITP-CE experiments were performed using 80 mM tris–chloride (pH 8.3) as the LE and 80 mM tris–butyrate (pH 8.3) as the TE. For cases on the right (b), sample mixtures were injected as 52 mm long zones, and the total length for free-solution ITP was 182 mm. The remaining portion of 470 mm long capillary (with 400 mm detection length) was filled with 3% linear polyacrylamide. Reprinted from van der Schans *et al.*[79] Copyright 1995, with permission from Elsevier.

in a "fair race" of analytes where each enters CE mode nearly simultaneously and where the rapid transition also results in relatively longer time (and separation lengths) during CE mode.

4.1.2 Limitations and practical considerations. Unlike other tITP-CE methods, tITP-CE induced by sieving matrix gradients is limited to separation of macromolecular analytes whose mobilities are effectively lowered by a sieving medium. Further, compared to other tITP-CE methods, the sieving matrix gradient approach requires additional steps involving *in situ* polymerization of a sieving matrix gel. We note that, such preparation of gels in the separation channel is no different from that required in conventional capillary gel-electrophoresis.[3] However, as is usual with CE of macromolecules, the difficulties involved in replacing and reusing gels can be avoided by using less viscous and easily replaceable polymer solutions, such as hydroxyethylcellulose (HEC) and polyvinylpyrrolidone (PVP) without compromising separation resolution.[3]

4.2 Changing mobility order using spatial gradient in pH

Coupling of ITP and CE can also be realized through pH induced variations in mobility of weak electrolyte species. The observable electrophoretic mobility of weak electrolyte species, termed effective mobility, is defined as the average of mobilities of all its ionization states weighted with corresponding ionization fractions. Since degree of ionization depends strongly on the dissociation constant of weak electrolyte species and the local pH of the solution, effective mobilities of weak electrolyte species can vary strongly with pH. For example, the effective mobility (μ_{eff}) of a weak monovalent acid with acid dissociation constant $\text{p}K_{\text{a},-1}$ depends on pH as,

$$\mu_{\text{eff}} = \frac{\mu_{-1}}{1 + 10^{\text{p}K_{\text{a},-1} - \text{pH}}}, \quad (4)$$

where μ_{-1} denotes the mobility when the weak acid is fully ionized. Similar expressions for effective mobility of multivalent species are given in Persat *et al.*[82] According to eqn (4), the effective mobility of a weak monovalent acid is highest when $\text{pH} \gtrsim \text{p}K_{\text{a},-1} + 1$, *i.e.*, when the weak acid is fully ionized. Whereas, the effective mobility of a weak monovalent acid is low when $\text{pH} \lesssim \text{p}K_{\text{a},-1} - 1$. eqn (4) also suggests that lowering or raising pH can reverse the inequality relating the effective mobilities of two weak acid species, provided that the weaker of the two acids (with higher $\text{p}K_{\text{a},-1}$) has higher fully ionized mobility (μ_{-1}) than the other. For example, tricine ($\mu_{-1} = -30 \times 10^{-9}$ m^2 V^{-1} s^{-1}, $\text{p}K_{\text{a},-1} = 8.15$) has effective mobilities of -26×10^{-9} and -5.5×10^{-9} m^2 V^{-1} s^{-1} at pH values of 9 and 7.5, respectively, whereas, a relatively strong acid species HEPES with $\text{p}K_{\text{a},-1} = 7.5$ and $\mu_{-1} = -23.5 \times 10^{-9}$ m^2 V^{-1} s^{-1}, will have effective mobilities of -23×10^{-9} and -11.75×10^{-9} m^2 V^{-1} s^{-1} at pH values of 9 and 7.5, respectively. Such a reversal of the mobility order of two species at different pH values can be used to couple ITP and CE. In the above example, tricine can be used as TE ion to focus HEPES in ITP at pH 7.5 as the mobility of the former is very low at that pH. However, if the ITP zones are suddenly titrated to pH 9, HEPES will lag behind tricine (TE) and separate in the TE zone. Similarly, ITP and CE can be coupled if titration of ITP zones to a different pH causes mobility of LE ions to drop below the mobility of focused analytes. In that case, the analytes will overtake the LE/TE interface and separate in the LE zone.

ITP zones can be titrated to different pHs by creating a spatial pH gradient and letting the focused analyte zones migrate through it. Here we present two methods for generating spatial pH gradients inside channel to titrate ITP zones to different pHs. The first method involves stationary, diffusive pH gradients using adjoining zones of a binary electrolyte titrated to different pH values, whereas the second method involves strong, self-sharpening, and migrating pH gradients across a counter-migrating ITP interface in bidirectional ITP.

4.2.1 Stationary, diffusive pH gradients using binary electrolytes. A stationary pH gradient in ITP can be generated by using two LE zones in tandem consisting of a strong acid (or base) for LE ions and titrated with varying concentrations of a common weak base (or acid). For example, if the LE ion is the chloride ion from 100 mM HCl, coupling two sequential LE zones of 100 mM HCl/300 mM tris and 100 mM HCl/120 mM tris will yield pH values of 8.5 and 7.5, respectively. The interface between such zones of a binary electrolyte consisting of a weak base and a strong acid (or weak acid and strong base) titrated to different pHs is stationary under an applied electric field, subject only to diffusive broadening[28] or dispersion due to bulk flow.[63,74] To couple ITP and CE, analyte zones are made to migrate from one LE to the other such that ITP focusing conditions do not hold at the pH of the second LE. This then causes analytes to defocus and separate in CE within the second LE zone.

In general, it is not practical to achieve more than 1 unit change in pH by titrating a strong acid (or base) with varying concentrations of a weak base (or acid), without sacrificing the buffering capacity of the electrolyte solution. Such small pH changes across stationary interfaces limit their applicability for inducing sufficient mobility change to couple ITP and CE. For this reason, mobility changes caused by stationary pH gradients have not been used as a standalone method of coupling ITP and CE. However, we note that stationary pH gradients have been used successfully in conjunction with a gradient in the sieving matrix concentration to change the relative mobility order of species in ITP and trigger CE. For example, Ornstein[28] and Davis[29] used a large-to-small gel pore size gradient to lower the mobilities of focused proteins and simultaneously employed a gradient in pH to increase the effective mobility of TE ions; this helped ensure that TE ions overtook the focused proteins and triggered CE separation.

4.2.2 Counter-migrating and self-sharpening pH gradients in bidirectional ITP. Bidirectional ITP offers a convenient way of focusing analytes *via* one ITP interface and simultaneously generating a strong pH gradient across a second, counter-migrating ITP interface. The method can be performed in a single, straight separation channel and the entire process (ITP preconcentration, transition to CE, and subsequent CE separation) is established by initial conditions. Interaction of focused analyte zones with the counter-migrating ITP interface results in a rapid exchange of counter-ions and a corresponding change in pH. For example, in anionic ITP, bidirectional ITP

can be used to rapidly change the local counter-ion to a new cation which has a significantly different acid dissociation constant (pK_a). Bidirectional ITP involves two sets of LE and TE ions corresponding to cations and anions. We term these LE+, LE−, TE+, TE− ions, where the + and − signs correspond to cations and anions, respectively. Bidirectional ITP can be used to create simultaneous anionic and cationic ITP interfaces (ion-concentration shock waves) which approach each other and interact.[42,83]

Recently, Bahga *et al.*[42] demonstrated for the first time that anionic and cationic ITP shock waves in bidirectional ITP can be made to approach each other and interact to effect strong changes in ITP conditions. For example, ITP focusing of anionic analytes can be disrupted by shock interaction, initiating a CE separation. The method demonstrated by Bahga *et al.*[42] was based on focusing anionic analytes at the anionic ITP shock (LE−/TE− interface) and simultaneously creating a sharp pH gradient across the counter-migrating cationic ITP shock (LE+/TE+ interface). When the focused analyte zones cross and interact with LE+/TE+ interface, TE+ ions replace LE+ ions as the counter-ion for anionic ITP. As a result, anionic analyte zones and the TE− are each titrated to a new pH determined by the newly arrived TE+. The process can be designed so that ITP focusing conditions no longer hold for analytes, and thereafter analytes defocus and separate according to their electrophoretic mobilities.

Bidirectional ITP can be used to focus and separate both anions and cations, albeit in separate experiments and/or channels. Also, disruption of ITP focusing of analytes following shock interaction can be achieved by one of two basic methods: ion mobility(ies) can be changed such that analyte ions overtake LE ions or such that TE ions overtake analyte ions. These cases result in CE separations of analytes within the LE or TE zones, respectively. Here, as an illustrative example, we limit our discussion to the focusing and separation of anionic analytes using a bidirectional ITP system where TE ions overtake analyte zones after the shock interaction. Fig. 10a–c show the schematics of bidirectional ITP experiments of Bahga *et al.*[42] for focusing and separation of anionic analytes. Initially, a single, straight channel is filled with a mixture of LE+, LE−, and anionic analytes S1− and S2−. The left (cathodic) reservoir is filled with anionic TE (LE+/TE− mixture) and the right (anodic) reservoir is filled with cationic TE (TE+/LE− mixture). The electrolyte chemistry is chosen such that mobilities of LE− and TE− ions bracket the mobilities of analyte ions, prior to shock interaction. When an electric field is applied, S1− and S2− focus between LE− and TE− zones and migrate towards the right, as shown in Fig. 10b. Simultaneously, a cationic ITP shock forms between LE+ and TE+ zones and migrates leftwards. Later, when the LE+/TE+ interface interacts with focused analyte zones, TE+ replaces LE+ as the counter-ion for anionic ITP. Here, we choose a TE+ ion with a higher pK_a than LE+, resulting in a higher pH and increased ionization of the TE− ions. This increases the mobility of TE− ions above those of S1− and S2− ions. Consequently, TE− ions overtake S1− and S2− zones and thus trigger CE separation of S1− and S2−, as shown in Fig. 10c.

A key requirement for the method shown in Fig. 10 is that the pH of analyte zones increases substantially as TE+ replaces LE+ as the counter-ion of anionic ITP zones. Not only should the LE+ be a weaker base than TE+ but LE+ should have a higher fully ionized mobility than TE+ to ensure the formation of a sharp, cationic ITP front (thus ensuring a rapid ITP-to-CE transition). A large increase in effective mobility of TE− ions upon shock interaction can be ensured if $pK_{a,LE+} < pK_{a,TE-} < pK_{a,TE+}$. Bahga *et al.* provided several choices for electrolyte chemistries, each satisfying these criteria. One of their electrolyte systems used chloride ions from hydrochloric acid as LE−, tricine ($pK_{a,TE-} = 8.15$) as TE−, imidazole as LE+ ($pK_{a,LE+} = 7.15$), and arginine as TE+ ($pK_{a,TE+} = 8.92$). Fig. 10d–g show simulation of focusing and separation of two anionic analytes (S1− and S2−) using this electrolyte chemistry. Here, the analytes S1− and S2− were assumed to be fully ionized with mobilities of -20×10^{-9} and -12×10^{-9} $m^2\ V^{-1}\ s^{-1}$, respectively. Fig. 10d and e show analytes S1− and S2− focused between LE− and TE− zones prior to shock interaction. Fig. 10f and g show analytes separating behind the LE−/TE− interface after the shock interaction. Note that the necessary condition for separation to occur after the shock interaction is that TE− ions should overtake the analyte ions. However, TE− ions need not overtake LE− ions, as is evident from the intact LE−/TE− interface speeding away from analytes to the right as shown in Fig. 10g.

Fig. 11 presents sample experimental results of Bahga *et al.*[42] showing preconcentration and separation of 11 ds-DNA fragments of a 1 kbp DNA ladder using bidirectional ITP. The electrolyte chemistry used in these experiments was similar to that used in simulations shown in Fig. 10. Under the conditions of the experiment shown in Fig. 11, mobilities of DNA fragments do not appreciably change after shock interaction (as DNA is well acidic). However, increase in TE− mobility after the shock interaction causes TE− ions to overtake focused DNA fragments and trigger CE separation. Fig. 11a shows all DNA fragments of the 1 kbp ladder focused within a narrow zone prior to shock interaction. ITP-to-CE transition occurs within a fraction of a second, and the initial phase of CE separation is shown in Fig. 11b. Fig. 11c shows a fully resolved DNA ladder consisting of 11 distinct peaks corresponding to 10, 8, 6, 5, 4, 3, 2, 1, 1.5, 1.5, 0.517, and 0.500 kbp fragments. The measured electropherogram is in qualitative agreement with slab-gel separations of the same DNA ladder (Fig. 11d). Note that, bidirectional ITP resolves peaks corresponding to 500 and 517 bp which are unresolved in the slab-gel electrophoresis data.

4.2.2.1 Advantages. Coupled ITP and CE using bidirectional ITP yields significantly higher resolution separations than tITP-CE methods based on disruption of the ITP zone order. In bidirectional ITP, shock interaction results in a sudden change in pH of the analyte zones which disrupts the required mobility order for ITP. As a result, analytes begin to separate immediately after the counter-migrating ITP shock rapidly washes over the focused analyte zones. This is in contrast to tITP-CE methods based on disruption of the ITP zone order (discussed in Section 3), where slow transition from discontinuous to uniform electrolyte system leads to gradual defocusing of analytes and correspondingly low separation resolution. Moreover,

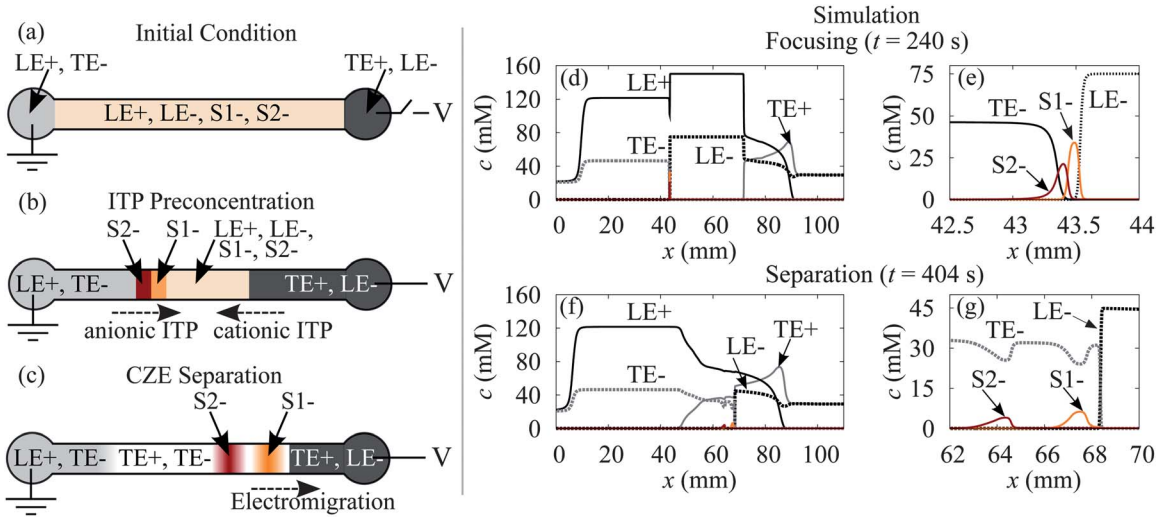

Fig. 10 Schematics (a–c) and simulation (d–g) illustrating ITP focusing and CE separation using bidirectional ITP. (a) Initially, channel is filled with a mixture of LE+, LE− and anionic analytes S1− and S2−. The left (cathodic) reservoir is filled with anionic TE (LE+/TE− mixture) and the right (anodic) reservoir is filled with cationic TE (TE+/LE− mixture). (b) When voltage is applied, S1− and S2− focus in narrow anionic ITP zones and migrate rightwards. Simultaneously, a cationic ITP shock forms between LE+ and TE+ zones and migrates leftwards. (c) When the cationic ITP shock (LE+/TE+ interface) interacts with focused analyte zones, TE+ replaces LE+ as the counter-ion for anionic ITP. This change in counter-ion species for anionic ITP in turn changes the local pH of anionic ITP zones. If the electrolyte chemistry is chosen correctly, the sudden change in pH after interaction of anionic and cationic ITP shocks can result in disruption of ITP focusing conditions for S1− and S2−. In the case depicted here, the pH change causes mobility magnitude of TE− to increase above those of analytes. TE− ions overtake S1− and S2− zones and result in electrophoretic separation of S1− and S2−. (d–g) Simulated concentration profiles before and after the interaction of anionic and cationic ITP shocks in bidirectional ITP. Plots (e) and (g) are detailed views of the species distributions shown in plots (d) and (f), respectively. (d and e) Prior to the shock interaction, analytes S1− and S2− focus in anionic ITP. Plot (d) shows rightward propagating LE−/TE− interface at $x = 43$ mm, and leftward propagating LE+/TE+ interface at $x = 70$ mm. (e) Detailed view of S1− and S2− focused at the LE−/TE− interface. (f and g) Separation of S1− and S2− after interaction of anionic and cationic ITP shocks. Plot (f) shows anionic ITP shock (LE−/TE− interface) at $x = 68$ mm, and dispersed LE+/TE+ interface at $x = 50$ mm. LE+/TE+ interface disperses because TE+ mobility becomes greater than LE+ mobility after the shock wave interaction. However, disruption of the leftward propagating cationic ITP interface does not interfere with CE separation of S1− and S2−. (g) Fully resolved peaks of S1− and S2−, and an intact LE−/TE− interface. LE+/LE− mixture was 150 mM imidazole and 75 mM hydrochloric acid; LE+/TE− mixture was 20 mM imidazole and 20 mM tricine; TE+/LE− mixture was 30 mM arginine hydrochloride. Anionic analytes S1− and S2− were assumed to be fully ionized and their mobilities were -20×10^{-9} and -12×10^{-9} m^2 V^{-1} s^{-1}, respectively. Simulations were performed for a constant current of 1.4 μA in a 74 μm wide and 12 μm deep D-shaped, wet-etched channel. Electroosmotic flow was accounted by assuming electroosmotic mobility of 2×10^{-9} m^2 V^{-1} s^{-1}. Reprinted with permission from Bahga *et al.*[42] Copyright 2011, American Chemical Society.

peak migration times in bidirectional ITP are directly correlated with analyte mobilities as all analytes defocus and start separating immediately after the shock interaction. This is in contrast to tITP-CE methods based on disruption of the ITP zone order where analytes transition from ITP to CE at varying times, depending on their mobilities.

Lastly, bidirectional ITP does not require specialized equipment, such as column-coupled channels or pressure controller for hydrodynamic counter-flow, to couple ITP and CE. Instead, bidirectional ITP can be applied for focusing and separation of analytes in single, straight channel systems (*e.g.*, with a uniform sieving matrix concentration), including standard electrophoresis systems. Also, transition from ITP to CE is fully automated *via* shock interaction and requires no intermediate steps, such as switching the electric field or replacing electrolytes between ITP and CE steps.

4.2.2.2 Limitations and practical considerations. The aforementioned advantages of using bidirectional ITP to couple ITP and CE, however, come at a cost of limited choices of electrolyte systems that can guarantee ITP focusing prior to shock interaction and subsequent CE separation. In contrast, virtually any ITP electrolyte system can be used for tITP-CE methods based on disruption of the LE-sample-TE zone order. For example, in the LE-sample-LE system discussed in Section 3.1.2, injecting LE ions behind the analyte and TE zone will inevitably disrupt ITP and trigger CE. However, shock interaction in bidirectional ITP does not necessarily trigger CE.[84] Moreover, due to the large pH variations required to trigger CE, we believe that bidirectional ITP may not be generally suitable for protein separations, as significant pH changes can lead to decreased solubility and reversal of the sign of charge on the proteins.

5 Conclusions

We have presented a comprehensive review and comparison of methods for online coupling of transient ITP and CE. Transient ITP preconcentration prior to CE separation significantly enhances detection sensitivity of CE. tITP-CE of trace analytes also improves separation resolution by providing an ideal sample injection for CE where analytes are delivered in a narrow zone composed of overlapping analyte peaks. To compare a diverse set of tITP-CE methods, we presented a framework to categorize all tITP-CE methods based on their fundamental principles of disrupting ITP and triggering CE. We showed that tITP-CE methods can be broadly classified into those where CE is triggered by: (1) disrupting the LE-sample-TE zone order of

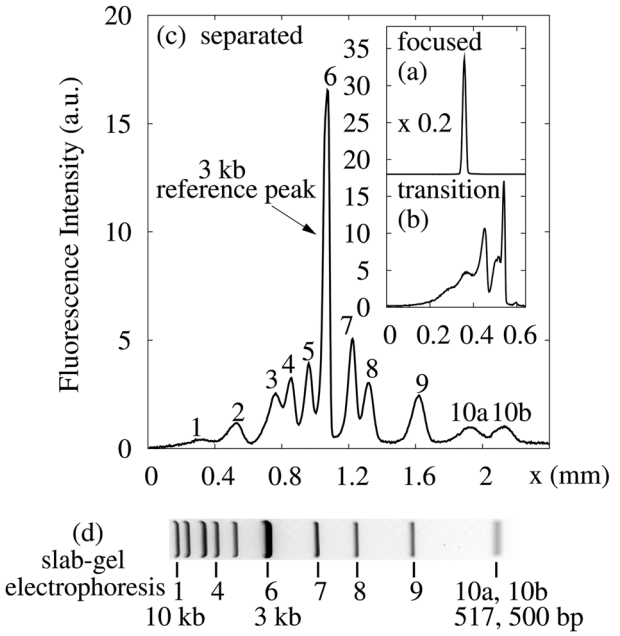

Fig. 11 Experimental results from Bahga *et al.*[42] showing measured fluorescence intensity during ITP focusing, and two instances of the CE separation of the 1 kbp ds-DNA ladder using bidirectional ITP. (a) Initially, all DNA fragments focus in a narrow anionic ITP zone. (b) The ITP-to-CE transition takes approximately 0.1 s, and DNA fragments begin separating just after the cationic ITP interface washes over the focused DNA fragments. (c) Electropherogram showing a fully resolved DNA ladder with distinct peaks corresponding to 11 fragments in the DNA ladder. The electropherogram was recorded 15 mm downstream of the point where anionic and cationic ITP shocks interacted. (d) Visualization (inverted) of agarose gel separation of a 1 kbp ds-DNA ladder (provided by the vendor, New England BioLabs, Ipswich, MA). Unlike bidirectional ITP, agarose gel electrophoresis does not resolve peaks 10a and 10b corresponding to 517 and 500 bp fragments. Reprinted with permission from Bahga *et al.*[42] Copyright 2011, American Chemical Society.

ITP or (2) changing the order of LE, TE, and analyte mobilities. Our review of various tITP-CE methods showed that each method has distinct advantages, trade-offs, and limitations. These considerations arise due to the differences in sample type, hardware, and/or electrolyte systems.

tITP-CE methods based on disruption of the LE-sample-TE zone order of ITP are applicable to a wide range of ionic species and do not require specialized electrolyte chemistry. However, transition from ITP to CE in these methods is gradual and depends strongly on the mobility of analytes and electrolyte chemistry. As a result methods based on disruption of the ITP zone order have typically low separation resolution, and the migration time of analyte zones cannot be used easily for peak assignments. In Section 3, we reviewed several methods which ensure faster ITP-to-CE transition during disruption of the ITP zone order. However, all those methods require specialized hardware, such as column-coupled channels or accurate external pressure controllers for applying hydrodynamic counter-flow. tITP-CE methods based on changing the mobility order of species (Section 4) enable rapid ITP-to-CE transition and yield high resolution CE separations in simple, straight channel systems. However, these methods are limited to a specific class of analytes and/or electrolyte systems. For example, tITP-CE based on the gradient in the sieving matrix is only applicable to macromolecular analytes, such as nucleic acids and proteins. Also, coupling ITP and CE using pH gradients is limited to those cases where the mobility order of LE, TE, and/or analyte ions varies considerably and differently with pH.

There is a wide variety of tITP-CE methods and each has unique tradeoffs between separation resolution, complexity of hardware, and applicability to different analytes. We offer our comparison of various tITP-CE methodologies as an aid to practitioners in selecting an optimal method for their requirements.

Acknowledgements

S.S.B. is supported by a Mayfield Stanford Graduate Fellowship and a Kodak Fellowship. This material is based upon work supported by the Defense Advanced Research Projects Agency under contract number HR0011-12-C-0080.

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