

On-Chip Million-Fold Sample Stacking Using Single-Interface Isotachophoresis

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ABSTRACT

We present a simple and robust isotachopheresis (ITP) method that can be integrated with microchip-based capillary electrophoresis (CE) devices to achieve million-fold sample stacking. We performed an experimental parametric study to show the effects of initial sample ion concentration, leading ion concentration, and trailing ion concentration on ITP stacking. We also discuss the usefulness and limitations of a simple one-dimensional non-dispersive model and a scaling analysis for dispersion rate. We found that a single-interface ITP configuration together with electroosmotic flow suppression and high leading ion concentration provide high performance ITP and can be integrated readily with CE separation. We demonstrated detection of trace of 100 fM alexa fluor 488 (signal-to-noise ratio of 11) with a concentration increase of a factor of $2E6$. Application of our ITP/CE protocol to the stacking and separation of negatively charged fluorescent tracers (alexa fluor 488 and bodipy) resulted in a concentration increase of $6.4E4$ and a signal increase of $4.5E5$. The ITP/CE protocol can be performed with a standard microchannel cross design or simple flow control. The method can be implemented with available off-the-shelf chip systems using off-the-shelf voltage control systems and buffer chemistries.

KEYWORDS

On-chip electrophoresis, isotachopheresis; capillary electrophoresis; sample stacking; microchip; high sensitivity

INTRODUCTION

On-chip capillary electrophoresis (CE) has been the subject of extensive research over the past decade.^{1,2} Microfabrication provides advantages over traditional capillary systems including rapid separation, reduced sample volumes, and integration with other microfluidic functions. However, the limit of detection (LOD) of on-chip CE systems can be limited by associated small sample volumes and the shallow depth of etched channels (typically 10-20 μm), which limits path length available to photodetectors. One way of improving sensitivity is to integrate an online sample stacking method. Stacking provides robust injection schemes since preconcentration reduces effective sample injection width, and stacking enables the use of less sensitive detection modes, such as electrochemical detection or UV absorption.³ On-chip stacking methods include electromigration-based processes such as field-amplified sample stacking,^{4,5} large volume sample stacking,⁶ and isotachopheresis (ITP);^{7,8} affinity-based sample stacking methods including sweeping⁹ and solid-phase extraction (SPE);¹⁰ and electrophoretic focusing methods such as isoelectric focusing (IEF)¹¹ and temperature gradient focusing (TGF).¹²

Electromigration-based sample stacking leverages spatial gradients of electrophoretic velocity of sample analytes as effected by gradients in ion density, mobility, and/or solvent viscosity. For FASS, the highest signal enhancement factor has been typically limited to 1,000-fold using free-standing capillaries,^{13,14} and 100-fold using microchips^{15,16} prior to our demonstration of 1000-fold on-chip FASS.¹⁷ (We now know the latter work was a hybrid stacking method that combined aspects of FASS with the mobility-based segregation dynamics of ITP.) The highest reported ITP signal enhancement factor has been roughly 500-fold for microchip experiments,^{18,19} and 5,500-fold for free-standing capillaries.²⁰ The latter is, to our knowledge, the highest demonstrated signal increase for a single step, electromigration-based preconcentration method prior to the current work. Million-fold signal increase was achieved by Quirino and Terabe,²¹ but by combining two sample stacking methods (FASI and sweeping) in series in a 10 min assay involving at least two manual buffer exchange steps. Very recently, Wang *et al.* reported million-fold stacking of GFP using so-called electrokinetic trapping caused by what they suggest is an extended space charge layer at the interface between micro- and nano-meter scale channels in a custom-built chip.²²

ITP is often combined with CE as a robust, pre-separation sample stacking method. Kendall and Crittenden²³ first demonstrated ITP using rare earth metals and simple acids.²⁴ ITP was first

implemented on chip by Walker and Morris.⁷ ITP uses multiple electrolytes where electrophoretic mobilities of sample ions are less than that of a leading electrolyte (LE) and greater than that of a trailing electrolyte (TE).²⁵ Individual species of the sample form narrow zones between the LE and TE and migrate with the same velocity (“isotacho” means equal velocity). With judicious choice of LE and TE chemistry, ITP is fairly generally applicable, can be accomplished with samples initially dissolved in either or both the TE and LE electrolytes, and (unlike FASS) does not require very low electrical conductivity background electrolytes. One disadvantage is that it requires some *a priori* knowledge of electrophoretic mobilities of sample ions.

In this paper, we describe a simple and robust method to integrate ITP with CE on a standard microfluidic chip. We demonstrate million-fold concentration increase in a fast, single step electromigration assay. We present an experimental parametric study of ITP to explore the effects of concentrations of LE, TE, and sample analytes on stacked sample concentration and peak width. We also compare these results with the trends predicted by a simple non-dispersive, closed-form, analytical model for ITP electromigration. Together, the results demonstrate a process by which to optimize ITP performance and predict trends. The results also provide validation data for more comprehensive models. Our ITP/CE injection, stacking, and separation method requires only a simple channel configuration, is performed with simple voltage control, and can be applied to off-the-shelf microchips.

THEORY

In the next two sections, we present a simple electromigration model and scaling arguments for dispersion rate. These simple formulations summarize the salient physics of ITP and were used here to deduce strategies for optimizing ITP processes. More comprehensive models of ITP that include electromigration and diffusion of multiple species (although not the effects of convective dispersion) are given by Schwer *et al.*²⁶ and Martens *et al.*²⁷

Non-dispersive 1D electromigration model

We here present an 1D non-diffuse ion species model for ITP focusing. Although simple, we shall see this model is useful in pointing out fundamental trends in ITP stacking and strategies for optimization. The general transport of ions is governed by a conservation law for dilute species,²⁸

$$\frac{\partial C_i}{\partial t} + \vec{u} \cdot \vec{\nabla} C_i = -\nu_i \vec{\nabla} \cdot (C_i \vec{E}) + D_i \nabla^2 C_i \quad (1)$$

where C_i is the molar concentration of ion i , ν_i is the electrophoretic mobility, and E is the electric field. We introduce the following scales for nondimensionalization: $[C] = C_0$, $[t] = \nu_0 E_0$,

$[\vec{u}] = -\epsilon \zeta_0 E_0 / \mu$, $[L] = w$, $[\nu_i] = \nu_0$, $[\vec{E}] = E_0$, $[D_i] = D_0$; where ν_0 , ζ_0 , D_0 , and δ are respectively the characteristic scales for electrophoretic mobility, zeta potential, diffusivity, and stacked sample zone length. The dimensionless equation is then

$$\frac{\partial C_i}{\partial t} + \alpha \vec{u} \cdot \vec{\nabla} C_i = -\nu_i \vec{\nabla} \cdot (C_i \vec{E}) + \frac{1}{Pe} D_i \nabla^2 C_i. \quad (2)$$

The parameters are defined as $Pe = E_0 \nu_0 \delta / D_0$ and $\alpha = -\epsilon \zeta_0 / (\mu \nu_0)$. Pe is the electric Peclet number, a measure of the ratio of diffusion time to electromigration time. α is the ratio of electroosmosis to electrophoretic mobility. Consider the case of large Pe and small α . This is the case of high E with suppressed electroosmotic mobility where both diffusion and advective dispersion are negligible compared to the effects of electromigration, so that

$$\frac{\partial C_i}{\partial t} + \nu_i \nabla \cdot C_i E = 0. \quad (3)$$

Multiplying Eq. (3) by z_i / ν_i and summing over all species

$$\frac{\partial}{\partial t} \left(\sum_i \frac{z_i C_i}{\nu_i} \right) + \nabla \cdot \left(E \sum_i z_i C_i \right) = 0 \quad (4)$$

where z_i is the valence number. The electroneutrality approximation requires the sum of products of concentrations and valences of ionic species must be zero so that $\sum_i z_i C_i = 0$. Eq. (4) then becomes

$$\sum_i \frac{z_i C_i}{\nu_i} = f(x). \quad (5)$$

This is the well-known Kohlrausch regulating function,²⁹ which suggests that sample concentration fields are constrained by the initial multi-species concentration profile.

We consider a model ITP system consisting of a leading ion, LE^- , sample ion, S^- , a trailing ion, TE^- , and common counter ion, A^+ (not shown), as shown in the schematic concentration plot Figure 1a (although here we consider only one sample ion). After ITP stacking, the interface between the sample and LE zones migrates at the same velocity so $\nu_{LE} E^R = \nu_S E^L$ (superscripts R and L denote the right-hand and left-hand zones, respectively). From electroneutrality, $C_A^R - C_{LE}^R = 0$ and $C_A^L - C_S^L = 0$, and electric current density, i , should be uniform along the direction of applied electric field: $i = \sigma^R E^R = \sigma^L E^L$. Here σ is the electrical conductivity of the solution, $\sigma = F \sum_i |\nu_i| C_i$. From Eq. 5 we obtain the following expression for concentration adjustment between the two zones in this simplified ITP process^{25, 30}

$$C_{S,final} = C_{LE}^R \frac{\nu_S (\nu_A + |\nu_{LE}|)}{\nu_{LE} (\nu_A + |\nu_S|)}. \quad (6)$$

Here, the adjusted sample concentration, $C_{S,final}$, is only a function of the LE concentration and electrophoretic mobilities (and not a function of, for example, TE concentration).

The degree of stacking in this simple ITP process can be characterized in terms of either concentration increase, CI or signal increase, SI . CI refers to stacked sample concentration divided by the original sample concentration. We use SI to refer to the ratio of the signal detected with sample preconcentration (e.g., at some downstream location after an electrophoretic separation) to the signal obtained without stacking.¹⁷ For the current model, $CI = C_{S,final}/C_{S,initial}$ where $C_{S,final}$ is given by Eq. 6. This suggests that CI increases in direct proportion with LE concentration and CI is (conveniently) inversely proportional to initial sample concentration. We shall see that both of these predicted trends are correct for realistic ITP conditions and so this analysis is useful in optimizing practical ITP processes. However, we shall also see that other observed trends, such as the dependence of stacked sample concentration on initial sample concentration (for fixed applied potential), are not captured by this simple model.

Practical ITP concerns: Scaling of dispersion rates

In this section we present some scaling arguments which, together with the simple model above, suggest strategies to optimize ITP in practice. The model presented above neglects the effects of molecular diffusion and convective dispersion on stacked sample concentration. (We here use the term dispersion to refer to both molecular diffusion and convective dispersion.) ITP has gradients of ion density and species mobility, which leads to axial gradients in electric field, and gradients of electrophoretic velocities of ionic species and electroosmotic velocity of bulk liquid.³¹ Electroosmotic velocity gradients are particularly important in determining dispersion. For any two regions of the channel, the electroosmotic velocity mismatch can be expressed as

$$\frac{U_{eo,1}}{U_{eo,2}} = \frac{\zeta_1 E_1}{\zeta_2 E_2} \cong \gamma^{-1+b}, \quad (7)$$

where $U_{eo,1}$ and $U_{eo,2}$ are the electroosmotic velocity of the two regions (e.g., in practical ITP, the

regions containing LE and TE). In each channel region, U_{eo} is determined by the applied electric field, local conductivity, and the local value of electroosmotic mobility. The rightmost term in Eq. 8 is one model for this velocity ratio where γ is the electrical conductivity ratio of the two zones, and b represents zeta potential (ζ) dependence on the concentration of electrolyte ($\zeta \sim C^b$) (the -1 term in the exponent captures the inverse dependence of conductivity on ion density). The value of b depends on the chemistry of the channel surface and the electrolyte solution; a typical range is between -0.2³² and -0.3.³³ This mismatch in EOF velocity generates a pressure gradient that causes sample dispersion and lowers stacking efficiency.³¹ These simple arguments suggest that EOF suppression is critical to optimizing the stacking efficiency of ITP.

In the limit of negligible EOF velocities (i.e., perfectly suppressed EOF), the dominant source of dispersion in ITP will be molecular diffusion. We can define a sample ion Peclet number in terms of the electrophoretic velocity of the sample and the width of the stacked sample zone,³⁴

$$Pe_s = \frac{E_\delta v_s}{D} \left(\frac{l}{CI} \right) \quad (8)$$

Here E_δ is the local electric field (in the stacked sample zone), D is the molecular diffusion coefficient of the sample ions, l is the effective initial length of the region of sample to be stacked, and CI is the stacking ratio, in general $CI = C_{S,final}/C_{S,initial}$. CI scales as l/δ , where δ is the length scale of the stacked sample zone. This Pe_s can be used to compare relative importance of electromigration (which leads to sample stacking) and diffusion (which works against sample stacking). Eq. 8 therefore suggests that large E_δ and large l lead to better stacking, two qualitative trends we observe in practice. E_δ is a function of applied field and the conductivities of the sample, LE, and TE zones. For example, for a given applied total potential, low TE concentration increases E_δ and should favor stacking.

l can be increased by injecting large, finite sample plugs. However, in practice we find it is even more effective to increase the effective value of l by setting up a single interface ITP process as shown in Figure 1a (inset). In this single interface configuration, the sample is initially uniformly dissolved into the TE and stacking is achieved in a zone between the TE and LE (see Figures 1b, c, and d). In this approach, the effective injected sample plug length is not limited by channel length but by the amount of sample that can flow into the channel (e.g., from the reservoir) and by the length between the injection region and the detector (which limits the time of stacking). In single interface ITP, sample concentration first increases and then saturates at a “fully stacked” value. The width of the stacked sample region then increases indefinitely (albeit very slowly). For low initial sample concentration and high LE concentration (the case of most interest), we can expect the local field in the TE zone to be a strong function of the (growing) length of the TE zone and TE ion concentration. The relatively low conductivity TE zone is a high resistance in series with the rest of the channel. We hypothesize that TE concentration and TE zone length also strongly affects the stacked sample zone electric field, E_δ .

EXPERIMENTAL

Materials and Instrumentation

N-hydroxyethylacrylamide (HEA) was purchased from Cambrex Bio Science (Walkersville, MD). V-50 initiator (2,2'-azobis (2-amidinopropane) dihydrochloride) was purchased from Wako Chemical USA (Richmond, VA). We synthesized polymers of HEA using free-radical polymerization in aqueous solution.³⁵ We used alexa fluor 488 and bodipy (Molecular Probes, Eugene, OR) as sample analytes. We used rhodamine B (Acros Organics, Morris Plains, NJ) as a neutral marker to quantify EOF. Sodium chloride, sodium hydroxide, and hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA). TE and LE consist of 5 mM HEPES (pH 7.0; Sigma, St. Louis, MO) titrated with sodium hydroxide and 1 M NaCl dissolved in deionized water, respectively. HEPES ions, chloride ions, and sodium ions were the trailing ion, leading ion and counter ion, respectively. We added 0.1 % w/v poly-*N*-hydroxyethylacrylamide (PHEA) to all electrolytes to suppress EOF.³⁵ All electrolyte solutions were filtered prior to use with 200 nm pore syringe filters (Nalgene Labware, Rochester, NY).

We used a standard, cross-pattern glass microchip (Micralyne, Alberta, Canada) with channels 50 μm wide and 20 μm in depth. Images of the sample injection, stacking, and separation process were captured with an inverted epifluorescent microscope (IX70; Olympus, Hauppauge, NY) with 4X (N.A. of 0.16), 10X (N.A. of 0.4), and 20X (N.A. of 0.5) objectives (Olympus, Hauppauge, NY) and an XF100-3 filter cube (Omega Optical, Brattleboro, VT) with peak excitation and emission wavelength ranges of 450-500 nm and 500-575 nm, respectively. Images were recorded using a generation III, intensified CCD camera (IPentaMAX; Roper Scientific, Trenton, NJ) with a 12-bit intensity digitization resolution. A LabVIEW-controlled high voltage power supply (Micralyne, Alberta, Canada) was used to control electric field for ITP/CE process.

ITP/CE protocol

We have developed a hybrid ITP/CE method for microchip-based CE devices. The injection method is depicted schematically in Figure 1. The four steps can be summarized as follows: (i) We perform a microchannel glass surface pretreatment where the entire microchannel is washed with 1.0 M HCl for 15 min. This is followed by flushing with 0.1% w/v PHEA solution for 15 min. (ii) Sample is loaded and the TE/LE interface formed. Here, a high conductivity LE buffer is loaded into the separation channel reservoir and a side channel reservoir (designated as east and north in the figure, respectively). A mixture of sample and low conductivity TE buffer is then loaded in the sample (west) reservoir. A single interface TE/LE boundary is formed at the cross-intersection of microchip by applying vacuum for 10 seconds at the sample waste (south) reservoir. (iii) The stacking step is next. Once the TE/LE boundary is established, a 2 kV electric potential is applied at the east reservoir and the west reservoir is grounded, forming an east-to-west electric field. This electric field initiates both electrophoretic migration of sample and trailing ions into the separation channel and ITP sample stacking. (iv) Separation and detection is achieved. Here, the electric field is switched toward the north reservoir by floating the west reservoir and grounding the north reservoir. In this key step, the ITP mode is terminated by injecting leading ions into the separation channel. Although the stacked sample zone is now well downstream of the intersection (typically 5 to 20 mm downstream), the leading ions overtake first trailing ions and then sample ions in the separation channel. This replacement of TE with LE effects an electrolyte exchange, terminating ITP stacking and initiating CE separation. Separated sample peaks are then detected downstream (using an epifluorescent microscope and a CCD camera). Our single-interface ITP/CE protocol is different than previous ITP/CE protocols in that the single-interface scheme provides a long effective injected sample plug length essential to achieve high sample stacking ratio, but also avoids manual buffer exchange steps (e.g., as employed by Xu *et al.*³⁶). The protocol is also accomplished with a standard four-well chip and without feedback control of injections (e.g., in contrast to the five-well chip and feedback control used by Wainright *et al.*³⁷).

Intensity normalization

The concentration of sample ions can change six orders of magnitude during the ITP stacking process. Since the dynamic range of CCDs or PMTs is limited, simultaneous detection of both the initial and stacked sample intensities is difficult in a single experiment. The dilute sample signal can be below the LOD of the system, while that of a stacked sample may saturate the detector. To accommodate this, we applied a quantitative (and calibrated) imaging technique. The measured sample concentration, C , is

$$C = C_{flat} \frac{I_{raw} - I_{bg}}{I_{flat} - I_{bg}} \quad (9)$$

where C_{flat} is the known concentration of highly concentrated sample analyte for the flatfield measurement, and I_{raw} , I_{flat} , and I_{bg} are respectively the signal intensities of stacked sample, flatfield, and background without sample (the latter with shutter open and illumination as normal). The flatfield image was obtained by imaging the microchannels filled with a homogenous concentration of dye with a molar concentration $1\text{E}3$ to $1\text{E}5$ times higher than (unstacked) initial sample concentration.

RESULTS AND DISCUSSION

To minimize dispersion due to EOF mismatch (see discussion of Eq. 7 above), we treated the microchannel walls with hydrophilic polymers. To validate this EOF suppression method, we measured electroosmotic mobility by injecting rhodamine B as a neutral dye. The electroosmotic mobilities of untreated and coated borosilicate microchannel were $5.9\text{E-}8\text{ m}^2/\text{Vs}$ and $2.0\text{E-}9\text{ m}^2/\text{Vs}$, respectively; so that the PHEA coating reduces EOF to 3.4 % of the untreated value.

We verified the dependence of signal intensity of the fluorophore on different background electrolytes and their pH values. We measured signal intensity of fluorescent sample analytes ranging from low concentration (1 nM) to high concentration (10 μM), each using the following solvents: deionized water (pH 5.4), TE (5 mM HEPES buffer, pH 7.0), and LE (1 M NaCl, pH 5.3). The measured signal intensity of the alexa fluor dye was linearly proportional to the sample concentration in all cases (typical regression coefficients were $R^2 = 0.9993$ to 0.9998) and varied by less than 5.0 % across solvents. The signal data can be interpreted as measurements of absolute sample concentration.

We tailored the voltage scheme of our ITP/CE method to our chip and detection system. Critical parameters in this customization include applied electric field strength (we limited this to values below which Joule heating was detectable from voltage versus current curves) and the duration of the stacking step (Step iii of the ITP/CE protocol). Figure 2 shows a schematic of typical curves of maximum concentration versus separation channel location for the current ITP/CE process. Several traces of actual data are overlaid for reference. In the ITP mode, sample peak signal intensity first increases approximately linearly and eventually saturates. Upon initiation of the CE mode, sample concentration scales as the square root of time (and $x^{0.5}$) due to diffusion. For the overlaid experimental curves, the applied field and initial sample concentration were 110 V/cm and 100 nM and the duration of stacking step was varied between 5 and 10 s. As shown, the maximum achievable concentration can be limited by the effective injected sample length as determined by the duration of the ITP step.

We performed an experimental parametric study to empirically optimize the ITP/CE process in our chip, demonstrate the strengths and weaknesses of the simple theory presented earlier, and provide validation data for future modeling efforts. We focused on the variation of three key parameters: the concentrations of LE, TE, and the initial sample concentration. We also performed some limited variations of injection time, detector location, and electric field.

We first summarize the variation of electric field, injection time, and detector location. In general, overly long duration of sample injection pushes the separation mode signal downstream of the detector (see Figure 2); while overly short sample injection durations terminate ITP mode before full sample stacking is achieved. The former adversely affects resolution while the latter lowers signal-to-noise ratio (*SNR*). Long sample injection leads to lower resolution since sample analytes have less time to separate before reaching the detector. Long sample injection therefore requires longer separation channel lengths and run times. For our cases of interest, we obtained good stacking and separation results for a 40 s sample injection/ITP duration with a nominal electric field of 220 V/cm and a detection point 30 mm downstream.

The effects of LE (NaCl) concentration, C_{LE} , TE (HEPES) concentration, C_{TE} and initial concentration of sample (alexa fluor), $C_{S,initial}$ on sample stacking are summarized in Figure 3. Fluorescent signals were detected with a viewing area 3.0 by 0.3 mm in the object plane at a point 30 mm downstream from intersection. All signals reached a fully stacked state (i.e., maximum concentration increase) within this distance from the intersection and during Step iii of the ITP/CE protocol. The exposure time and frame rate were respectively 10 ms and 20 frames per second. All cross-section-area-averaged axial intensity profiles were normalized as per Eq. 9.

C_{LE} was first varied from 10 mM to 1M to study its effect on maximum stacked sample concentration, $C_{S,final}$ and CI (Figure 3a). The error bars in this and Figure 4 below reflect 95% confidence intervals as determined from three realizations of each condition. Here, TE was fixed at a nominal value of 5 mM HEPES. The TE solution contained 1 nM alexa fluor 488 as a sample analyte.

The associated LE-to-TE conductivity ratios were between 13.9 and 1.21E3. The stacked sample concentration is nearly directly proportional to the concentration of LE ($R^2 = 0.95$ for a linear fit), as expected from the non-dispersive 1D model.

Figure 3a also shows the effect of C_{TE} on ITP stacking where we varied C_{TE} from 1 mM to 100 mM. The LE was fixed at 1 M NaCl, and $C_{S,initial}$ was fixed at 1 nM. The associated LE-to-TE conductivity ratios were between 66.2 to 6.01E3. Recall that the non-dispersive 1D model suggests that stacked sample concentration is not a function of $C_{S,initial}$ or C_{TE} . However, the measurements show that $C_{S,final}$ increased for lower C_{TE} (i.e., as conductivity ratio increases). We attribute this to the dependence of local field in the TE zone and stacked sample on TE concentration and the effects of dispersion. High LE-to-TE conductivity ratios (associated with low TE concentrations) increase the electric fields in the TE zone and the stacked sample zones. High electric field in the TE zone leads to fast stacking dynamics as there is a high electrophoretic flux of sample from the TE zone to the stacked sample zone. We hypothesize that high E_δ leads to high electric Peclet numbers (Eq. 8) and therefore high $C_{S,final}$, as the stacking process is less susceptible to dispersion.

Next, we varied $C_{S,initial}$ from 1 pM to 1 nM as shown in Figure 3b. The LE and TE were respectively fixed at 1 M NaCl and 5 mM HEPES. The LE-to-TE conductivity ratio was kept constant at 1.27E3 (113 mS/cm for LE and 89.0 μ S/cm for TE) for all cases in an effort to decouple the results from the effect discussed above (i.e., the dependence of the TE zone electric field and E_δ on LE-to-TE). Note that the effect of sample ions on electrical conductivity of the TE/sample mixture was negligible as $C_{S,initial} \ll C_{TE}$. The data shows CI increases as $C_{S,initial}$ decreases. This result is consistent with the 1D non-dispersive model. However, note that the dependence of CI on $C_{S,initial}$ is weaker than the inversely proportional dependence predicted by the simple model (CI changes just under two orders of magnitude while $C_{S,initial}$ changes three orders of magnitude). This discrepancy is also apparent in the measurements of the maximum concentration, $C_{S,final}$. The model predicts that $C_{S,final}$ should not be a function of $C_{S,initial}$. However, the experimental data shows that $C_{S,final}$ is a linear function of (although not directly proportional to) $C_{S,initial}$. This result is not attributable to changes in local field in the TE zone. Note that all measurements shown in Figure 3 were obtained in the fully stacked zone (e.g., flat region of the top curve of Figure 2). Clearly, more comprehensive models are required to capture the complexity of the current single-interface ITP stacking dynamics.

The width of sample analyte peaks also strongly depends on LE and TE concentration, and such data is important for validation of models of ITP which include both diffusion and advective dispersion effects. Figure 4 shows plots of the characteristic width, δ , and area of sample peaks, A_p , for various LE and TE concentrations. We characterized a peak width as twice the standard deviation of a fitted Gaussian. Peak area was estimated by multiplying peak width by peak heights and is a measure of the amount of sample in the peak. Here, we varied C_{LE} from 30 mM to 1M as shown in Figure 4a. TE and sample were fixed at 5 mM HEPES and 1 nM alexa fluor 488, respectively. δ decreased as C_{LE} increased. This is consistent with the results of Figure 3 (and with the simple model) since higher C_{LE} allows larger stacking ratio and so sample analytes can be focused more narrowly. Figure 4a also shows that A_p also increased as C_{LE} increased. For the single-interface ITP processes used here, the sample width is expected to increase indefinitely during the ITP step as more sample ions are transferred from the zone of TE/sample mixture to the sample zone. We hypothesize that, for higher C_{LE} values, a higher fraction of the total voltage drop is taken along the length of the TE region, increasing the flux of sample into the sample zone. This causes the peak to have lower migration velocity and higher stacking ratio. For increased C_{LE} , therefore, peak widths have a longer time to grow before being detected at the (fixed) detection point 30 mm downstream. This hypothesis is supported by an analysis of the transient data and by (full-field CCD image) measurements performed at a location just 1.5 mm downstream of the injector region. The latter observations show that lower C_{TE} and higher C_{LE} each result in larger slopes in curves of maximum (stacked zone) concentration versus separation channel distance, x . This data also suggests that lower C_{TE} cases reach the fully-stacked state at relatively short distances, while higher C_{LE} cases reach the fully-stacked state at long distances.

Next, we varied C_{TE} from 3 mM to 100 mM, while LE and sample were fixed at 1 M NaCl and 1 nM alexa fluor 488, respectively. As shown in Figure 4b, δ decreased as C_{TE} increased. Higher C_{TE} (with fixed C_{LE}) implies higher conductivity in the TE/sample zone. This lowers the TE/sample zone electric field and decreases the rate of stacking, increasing the time (and distance) to reach the fully-stacked state. For the fully-stacked states shown in Figure 4, therefore, the widths of the sample zone are narrower for high C_{TE} as the sample peak has had less time to accumulate width. This effect and the lower final concentration value (see Figure 3b) result in lower peak areas for higher C_{TE} .

We also demonstrated the detection of a trace analyte using ITP stacking alone. Figure 5a shows a spatiotemporal intensity plot of CI versus separation distance and time from which sample peak reaches the detection window, for an initial sample concentration of 100 fM of alexa fluor 488 dye. The LE was 1 M NaCl, and the TE was 5 mM HEPES buffer. We used a 10X objective (N.A. of 0.4) with viewing dimensions of 1.2 by 0.1 mm in the object plane. A brief (~ 2 min) sample stacking step enables the detection of 100 fM analyte concentration with $SNR = 11$. Measured concentration distributions yield a maximum measured concentration increase, CI , of approximately $2E6$ -fold. To our knowledge, this is the highest sample preconcentration for either free-standing capillary or on-chip electrophoresis systems.

Lastly, we demonstrated the effectiveness of the ITP/CE protocol with separations of alexa fluor 488 and bodipy. Figure 5b shows the separations of sample analytes detected 30 mm downstream of the intersection without (inset) and with ITP stacking. The electropherograms are determined by spatially integrating full-field CCD imaging data over a 60 by 60 μm region centered on the channel centerline to simulate the detection of a point-wise photodetector. The exposure time and CCD frame rate were 10 ms and 50 frames per second, respectively. The inset of Figure 5b shows the separation without ITP with initial (relatively high) concentrations of 100 nM and a uniform background electrolyte consisting of 5 mM HEPES buffer (pH 7.0). Signal intensity was normalized using the flatfield image signal of 100 nM alexa fluor 488. The applied field was 280 V/cm. The $SNRs$ of alexa fluor 488 (first peak) and bodipy (second peak) are 12.9 and 10.8, respectively, and the peak resolution is 29.4. We define SNR as the ratio of peak intensity to twice the standard deviation of background noise; and resolution as the distance between two peaks divided by the full-width half-maximum of the wider peak.³⁸ We then performed separation of this sample mixture diluted by a factor of $1E5$ (1 pM solutions each of alexa fluor 488 and bodipy) and applied the current single-interface ITP/CE method. The result is shown in Figure 5b. The injection/ITP time was 40 s and we used a nominal applied field of 220 V/cm. The resolution of these peaks is 6.1 and the $SNRs$ are respectively 57.6 and 39.0 for alexa fluor 488 and bodipy. This experiment achieves $CI = 6.4E4$ for alexa fluor 488. The ITP/CE method results in a $4.5E5$ -fold increase in signal relative to the unstacked case ($SI = 4.5E5$).

SUMMARY

ITP provides self-sharpening peaks and can be integrated with CE separation. An 1D non-dispersive model and scaling of dispersion analysis yields important insight into key ITP stacking parameters and suggests strategies for optimizing ITP in practice. These strategies include using high LE concentration and low initial sample concentration to maximize achievable concentration increase; suppression of EOF to minimize dispersions; and implementation of a single-interface configuration to inject a large effective sample width. We have implemented these strategies in the design of a single-interface ITP stacking method that combines a simple and robust ITP stacking step with a subsequent CE step. We first injected a sample/TE mixture to initiate ITP stacking and then injected LE at the channel intersection. The latter LE ion stream overspeeds TE ions behind the stacked sample zone, terminates ITP, and initiates CE separation.

We performed an experimental parametric study focused on the variation of LE, TE, and initial sample concentration. Consistent with the 1D non-dispersive model, we found that stacked sample concentration is proportional to the LE concentration, and the concentration increase, CI , (conveniently) increases as the initial sample concentration decreases. We also found that the stacked sample

concentration is a strong function of the TE concentration and the initial sample concentration. For our configuration, low conductivity TE zones result in higher TE zone electric fields and therefore faster rates of stacking. High TE zone fields are probably also correlated with high stacked zone fields, E_{δ} . We hypothesize that this, combined with the large effective injection length of the single-interface configuration, results in large sample electric Peclet numbers and efficient stacking that is less susceptible to dispersion. Results suggest that comprehensive multi-species models with coupled fluid flow, current conservation, and convective-diffusion-electromigration conservation are needed to fully describe ITP. Detection of trace of alexa fluor 488 was demonstrated with a maximum stacking ratio of 2E6-fold in 40 s and an LOD of 100 fM ($SNR = 11$). We also analyzed the separation of alexa fluor 488 and bodipy using our ITP/CE protocol, and demonstrated a CI of 6.4E4-fold and signal increase of 4.5E5-fold (in a 160 s ITP/CE process). Future work will include the formulation and validation of more comprehensive models of ITP for the single-interface configuration proposed here and further applications of the single-interface ITP/CE protocol.

ACKNOWLEDGEMENT

This work was sponsored by an NSF PECASE Award (J.G.S., award number NSF CTS0239080) with Dr Michael W. Plesniak as award monitor. The authors thank Tarun Khurana for insightful discussions regarding the physics of ITP. B.J. would like to thank Jung-Min Kee for technical assistance with the polymerization process.

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FIGURE CAPTIONS

Figure 1

Schematic of ITP/CE assay protocol. Configurations of co-ions are also shown at each step. (a) The north and the south reservoirs are filled LE, and the west reservoir is filled with a mixture of TE and sample. TE/LE boundary is formed by applying vacuum at the south reservoir. White arrows show the direction of pressure-driven flows. (b) ITP stacking is initiated by applying high voltage and ground at the east and west reservoirs, respectively. The black arrow denotes the direction of electric field. Sample anions electromigrate toward the anode as EOF is suppressed. The early stage of ITP stacking results in a partial separation (i.e., moving boundary electrophoresis). (c) The field is switched toward the north reservoir to inject LE ions behind the sample and initiate CE. ITP stacking continues until LE ions overtake the TE and sample ions. (d) Separation of samples occurs further downstream where sample ions electromigrate in nearly homogeneous LE electrolyte (remnant of TE not shown).

Figure 2

Schematic and various measurements of typical signal intensity changes in ITP/CE. Three specific signal intensities points are shown: The original, upstream sample intensity, a , the intensity of analyte detected at the downstream for ‘no stacking’ conditions, b , and the intensity of the stacked analyte detected at the downstream, c . Two figures of merit are the ratios c/a and c/b , which are respectively the concentration increase, CI , and the signal increase, SI . Sample injection time was varied from 5 to 10 s. LE and TE were respectively 50 mM NaCl and 5 mM HEPES. The potential drop between the electrodes during stacking process was 1 kV (nominal electric field of 110 V/cm). We used 2X objective (N.A. of 0.08) with viewing area of 6.0 by 0.1 mm in the object plane.

Figure 3

Parametric variations of initial concentration profile. The nominal applied field was 220 V/cm. CCD viewing area was centered 30 mm downstream of the channel intersection. (a) Maximum stacked sample concentration, $C_{S,final}$, versus LE concentration, C_{LE} and TE concentration, C_{TE} . For variation of C_{LE} , sample analyte and TE were respectively 1 nM alexa fluor 488 and 5 mM HEPES. The regression coefficient, R^2 , is 0.95. For variation of C_{TE} , sample analyte and LE were respectively 1 nM alexa fluor 488 and 1 M NaCl. $R^2 = 0.97$. (b) CI and $C_{S,final}$ versus initial sample concentration, $C_{S,initial}$. TE and LE were 5 mM HEPES and 1 M NaCl, respectively. The regression coefficients for CI and $C_{S,final}$ are respectively 0.97 and 0.98.

Figure 4

Plot of width, δ , and area, A_p , of stacked sample peaks. We characterized δ and A_p as respectively twice of the standard deviation of a fitted Gaussian, and the product of peak width and peak height. Sample was 1 nM alexa fluor 488. (a) δ and A_p as a function of C_{LE} with TE fixed at 5 mM HEPES. (b) δ and A_p as a function of C_{TE} with LE fixed at 1 M NaCl.

Figure 5

Demonstration experiments for ITP and ITP/CE protocol processes. (a) Detection of alexa fluor 488 at an initial concentration of 100 fM greater than million-fold concentration increase. LE and TE were respectively 1 M NaCl and 5 mM HEPES. We used a 10X objective (N.A. of 0.4). (b) Comparison of ITP/CE and CE separations of alexa fluor 488 (first peak) and bodipy (second peak). The position of the detector is 30 mm downstream of injector region. A 20X objective (N.A. of 0.5) was used. Electropherogram of separation with no stacking (inset) and 100 nM initial concentrations of alexa fluor 488 and bodipy. 5 mM HEPES used as a background electrolyte and applied electric field was 280 V/cm. For ITP/CE mode (main plot), initial concentrations of alexa fluor 488 and bodipy were both 1 pM in 5 mM HEPES (i.e., diluted by a factor of $1E5$). Here, LE was 1 M NaCl and nominal

applied field was 220 V/cm. The concentration increase, CI , and signal increase, SI , for alexa fluor 488 (first peak) are 6.4E4 and 4.5E5, respectively.

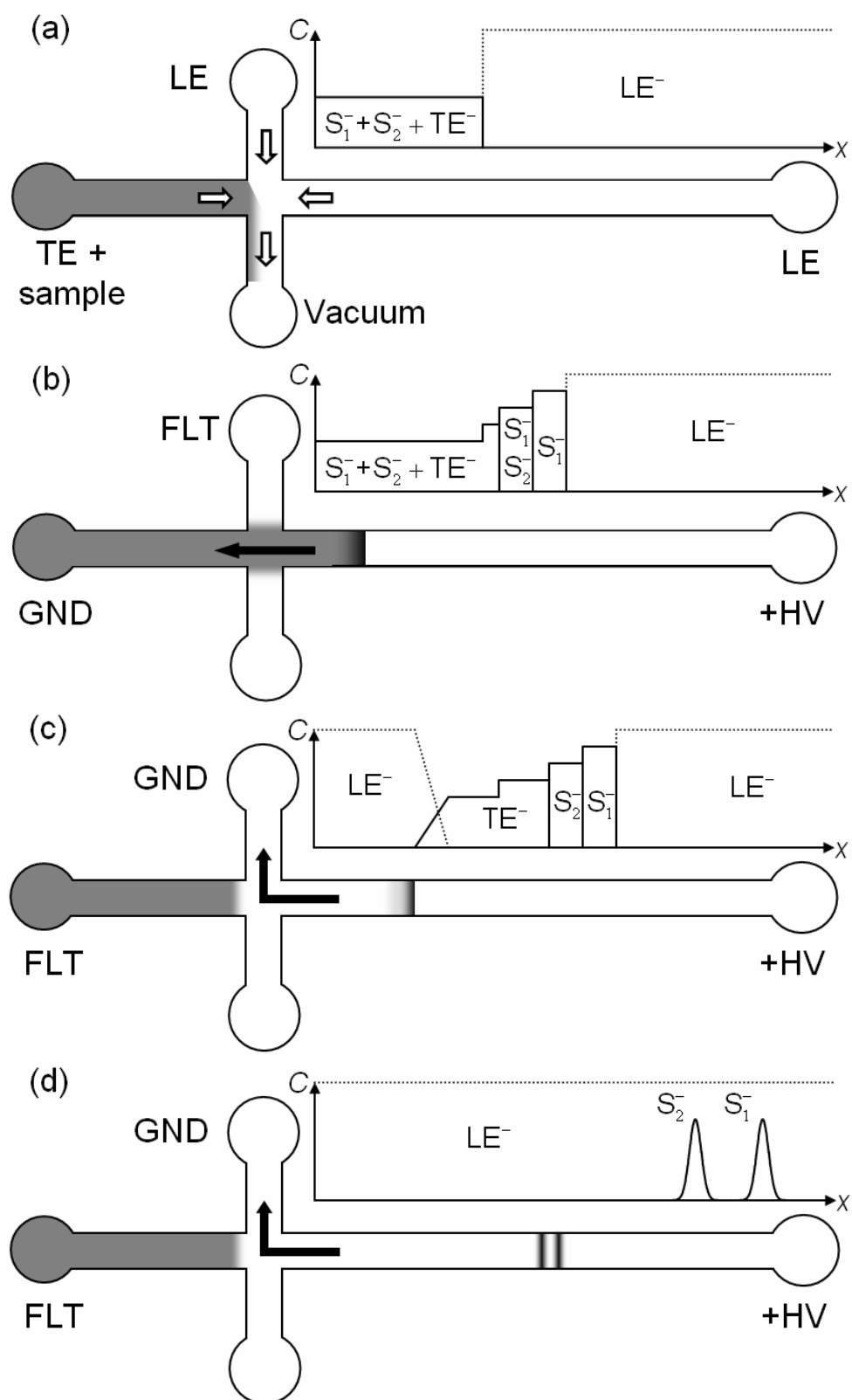


Figure 1

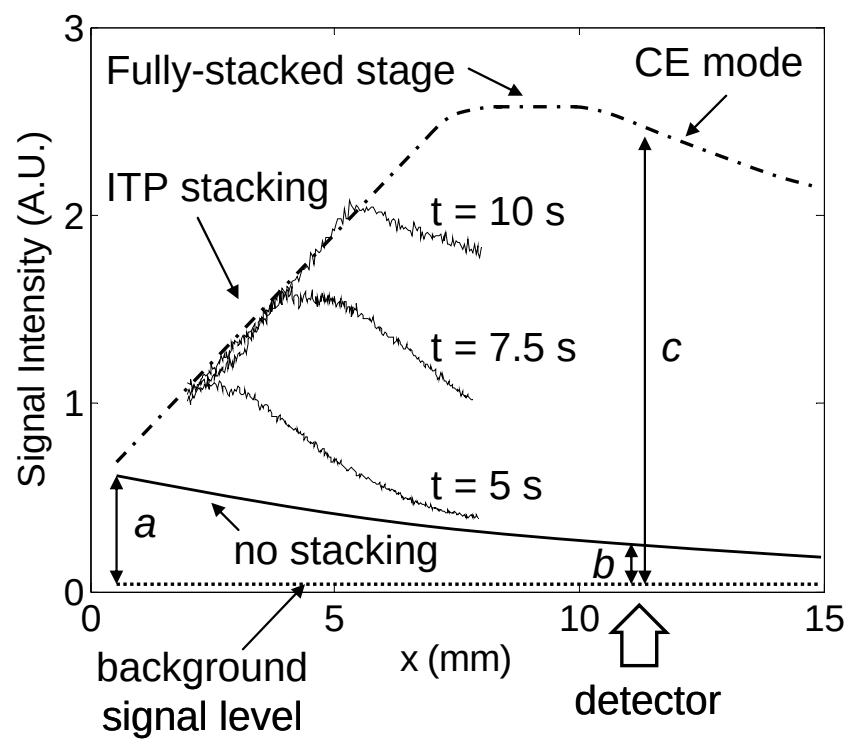
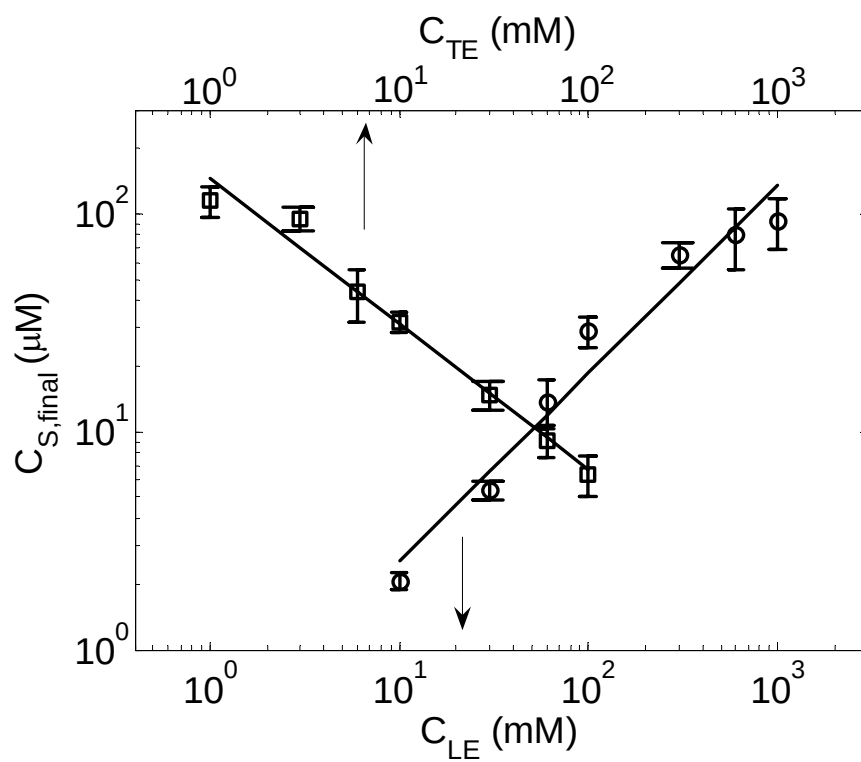
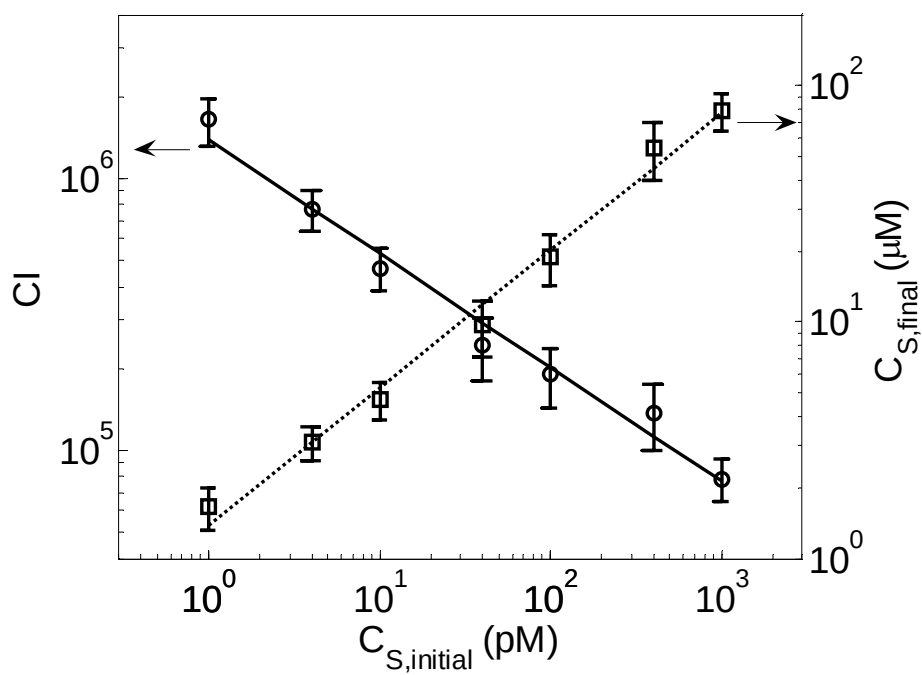


Figure 2

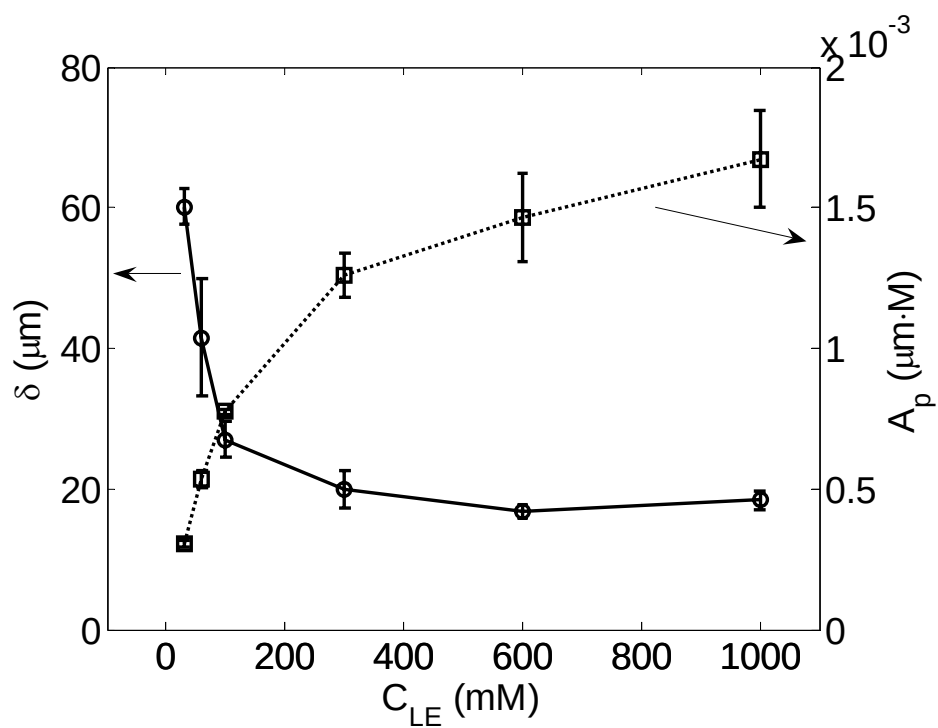


(a)

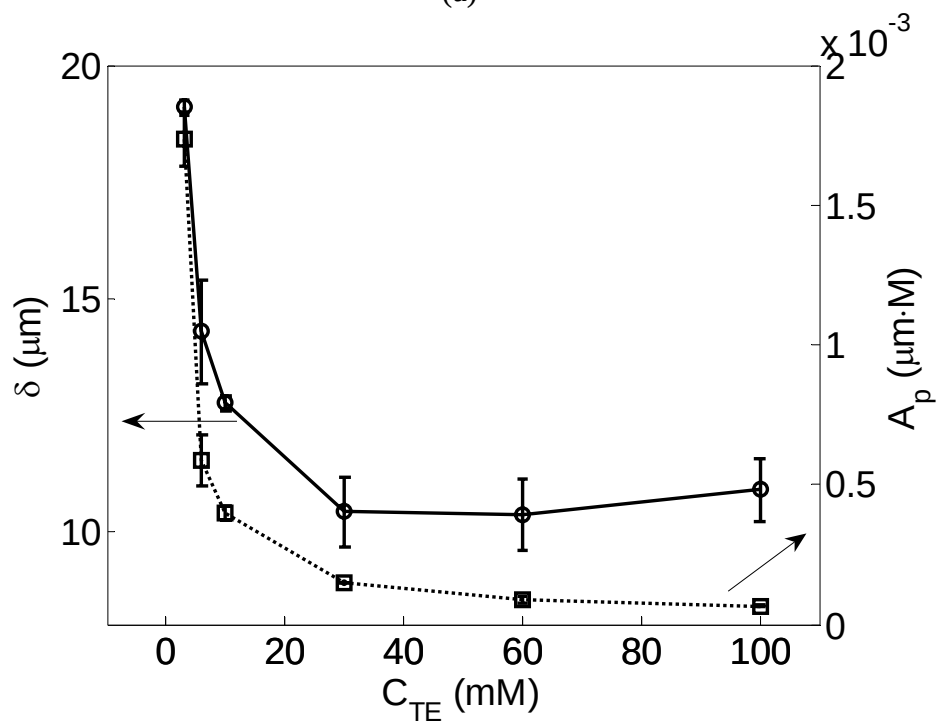


(b)

Figure 3



(a)



(b)

Figure 4

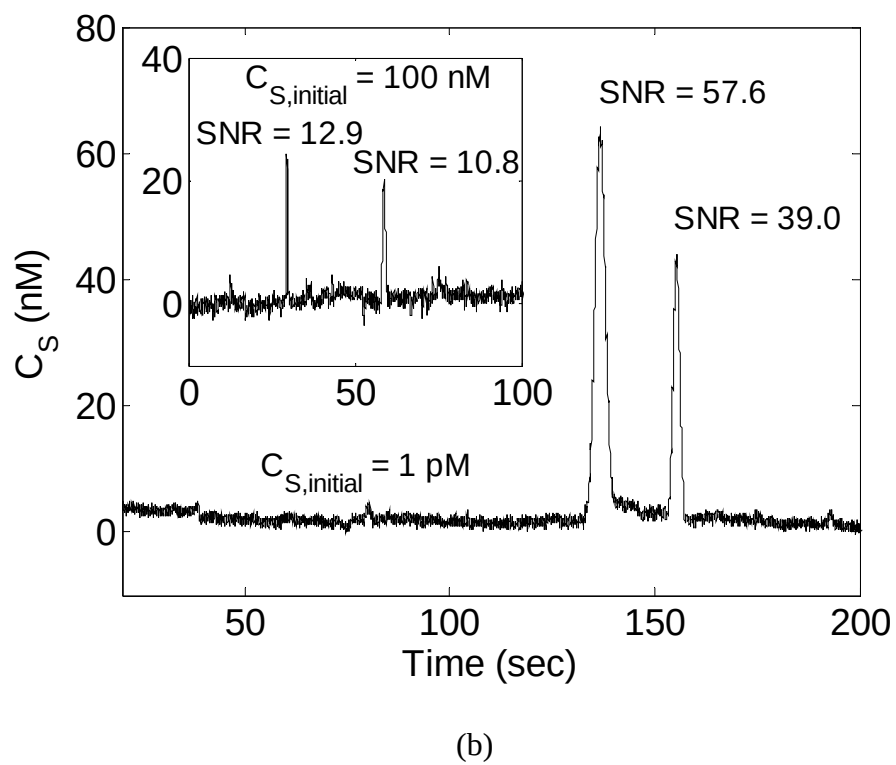
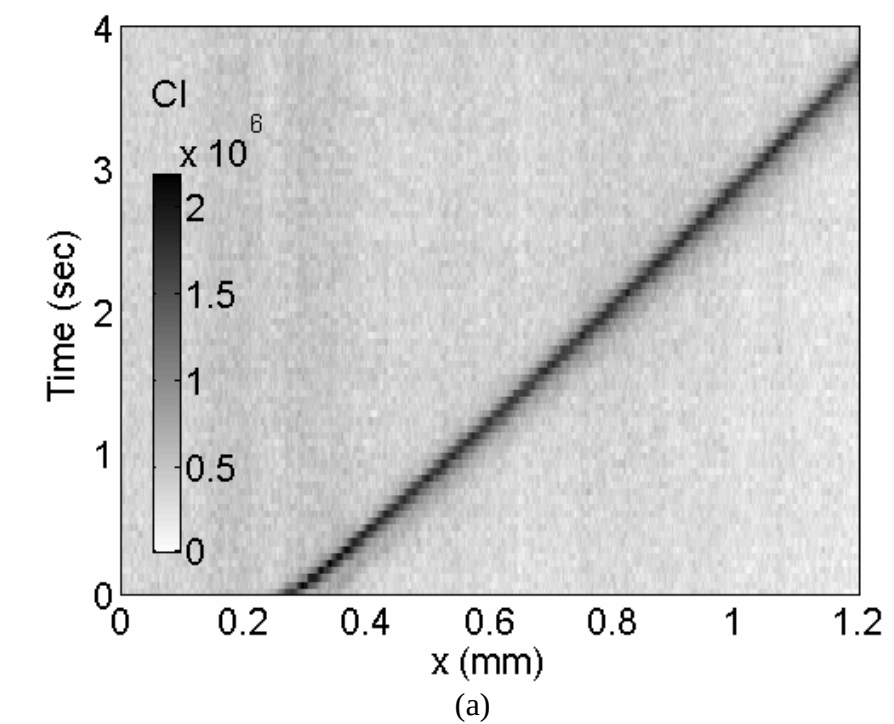


Figure 5