Transient Isotachopheresis Focusing of DNA and DNA-Protein Complexes Is Essentially Enhanced by Spontaneously Dissolved Aerial Carbon Dioxide in Electrolytes

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S Supporting Information

ABSTRACT: The formation of a highly adapted high-E zone is critical to isotachopheresis separation and focusing. Recently, we discovered that the high-E zone is present only in a small portion of electrophoresis channel in the presence of EOF (Liu, S. Q. et al. *J. Am. Chem. Soc.* **2013**, *135*, 4644–4647). Accordingly, a much narrower high-E zone is presumably present in t-ITP. If so, it is hard to achieve efficient t-ITP focusing. Indeed, by online coupling t-ITP with CE-LIF immunoassay, the immunocomplexes of carcinogenic BPDE-dG adducts are not efficiently focused using a freshly prepared background electrolyte. Intriguingly, we observed that 20-day stored background electrolyte displays a 10-fold better focusing efficiency. We hypothesize that the unexpected phenomenon is associated with the dissolution of aerial carbon dioxide, which is mainly converted to ionic HCO_3^- in the weak alkaline background electrolyte. Consequently, HCO_3^- of high electrophoretic mobility will be continuously injected into the capillary along with the background electrolyte and act as an alternative leading ion to improve the focusing. By addition of dry ice (without causing significant pH decrease, $\Delta\text{pH} < 0.4$) to freshly prepared background electrolytes, we immediately observed the enhanced focusing of immunocomplexes of the DNA adducts. NH_4HCO_3 and Na_2CO_3 , included in the background electrolyte, also improve the focusing efficiency and reproducibility. All these consistently support our hypothesis. To understand the underlying mechanism, an advanced CE-SMFI was exploited to monitor in real time the motion of single DNA molecules and the E change throughout t-ITP. We uncovered that t-ITP can induce a local high-E zone, but the presence of HCO_3^- in the background electrolyte could greatly increase the E value in the high-E zone, which allows more DNA molecules to rapidly move backward and to be efficiently stacked at LE/TE boundary. This study provides new insight into nonuniform electric field-induced electrophoresis focusing.

Isotachopheresis (ITP) has been widely applied for separation and preconcentration of DNA,^{1,2} proteins,^{3–5} as well as charged small molecules.^{6,7} To simplify the applications, a variant of ITP, transient ITP (t-ITP), is often used instead.^{8–11} By the use of discontinuous buffer, it is convenient to generate ITP and t-ITP formats coupling with other electrophoresis formats such as gel electrophoresis,^{12–14} capillary electrophoresis,^{15–21} and chip electrophoresis.^{22,23} In these applications, no special instrument is required.

Basically, ITP can be constructed by the use of two distinct electrolytes containing leading and terminating ions with highest and lowest electrophoretic mobility, respectively. The samples are electrokinetically or hydrodynamically injected into electrophoretic channel by being placed between the leading (LE) and terminating (TE) electrolytes.^{24,25} Ideally, target ions are focused at the LE/TE boundary while the leading and terminating ions are migrating at same speed by self-adapting electric field distribution. Surprisingly, we found that the initial TE zone of constant electric field strength (E) is split into two zones: a highly adapted zone of high-E value and a low-E zone

of gradual adapting electric field, in the presence of electroosmotic flow (EOF).²⁶ The E values of LE zone remain low, which is consistent with literature.^{27–29} Essentially, the highly adapted zone of the high-E value that is critical for ITP focusing only accounts for about $1/9$ to $1/5$ of the TE zone. This is achieved by continuous injection of LE.²⁶ It is reasonable to predict that the reduced injection volume of LE should reduce the volume of the highly adapted zone of the high-E value, too. Therefore, an issue is raised regarding how a transient ITP (t-ITP) focuses the sample ions by a very short pulse injection of LE or TE.

On the other hand, although t-ITP is also often used for effective electrophoretic focusing of a multitude of charged analytes in the presence of EOF,^{8–11} the detailed study on the dynamics and the underlying mechanism of t-ITP focusing is lacking.

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To clarify the above issues, we examined the t-ITP focusing of DNA-protein complexes. Unexpectedly, we found that the dissolution of aerial carbon dioxide in electrolytes essentially changes the t-ITP format and enhances the electrophoresis focusing of DNA and DNA-protein complexes. By the use of advanced capillary electrophoresis coupled single molecule fluorescence imaging (CE-SMFI), we further showed the dynamics of t-ITP and the real time evolution of electric field of t-ITP.

■ EXPERIMENTAL SECTION

Caution: ($\pm$)-anti-BPDE is carcinogenic and should be handled with extreme caution.

Materials and Chemicals. ($\pm$)-anti-Benzo(a)pyrene-trans-7,8-dihydrodiol-9,10-epoxide (($\pm$)-anti-BPDE) was supplied by the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Kansas, MO). Mouse monoclonal anti-BPDE-dG antibody (mAb 8E11) was obtained from Trevigen (Gaithersburg, MD). Alexa Flour 546 labeled goat antimouse IgG₁ fragment Fab, bovine serum albumin (BSA), and YOYO-1 iodide were purchased from Invitrogen (Carlsbad, CA). Calf thymus DNA (3.5 kb) was obtained from Merck (Whitehouse station, NJ). Ultrapure water was prepared using a Purelab Ultra Bioscience water purification system (ELGA, U.K.). pH meter was purchased from Mettler Toledo (Columbus, OH), and the conductivity meter was supplied by Shanghai Leici Instrument Factory (Shanghai, China). All other reagents were of analytical or HPLC grade.

($\pm$)-anti-BPDE Treatment. Mouse embryonic stem cells (about 5×10^6 cells per plate) were treated by 100 nM ($\pm$)-anti-BPDE for 2 h. In these cases, 2.5 μ L of dimethyl sulfoxide (DMSO) facilitating the dissolution of ($\pm$)-anti-BPDE was included in each plate. Therefore, a negative control was set by addition of only 2.5 μ L DMSO. Genomic DNA from the harvested cells was extracted by the use of Genomic DNA purification kit (Promega, Madison, WI) following the manufacturer's instruction. The concentration and quality of DNA were estimated by a Nanodrop-2000 UV-vis spectrophotometer (Thermo scientific, Waltham, MA) with the UV absorbance at 260 and 280 nm.

Capillary Electrophoresis-Laser-Induced Fluorescence (CE-LIF) Immunoassay. The CE-LIF immunoassay of the BPDE-dG adducts was conducted on laboratory-built CE-LIF instrument as described previously.¹⁹ Genomic DNA at 5.0 μ g/mL was denatured by heating at 95 °C for 5 min, and chilled on ice for 10 min to prevent denatured genomic DNA from reannealing. Then, it was mixed with 0.25 μ g/mL mouse monoclonal anti-BPDE antibody 8E11, 1.5 μ g/mL Alexa Flour 546 labeled goat antimouse IgG₁ fragment Fab (secondary antibody-2°Ab), and 100 μ g/mL BSA. The addition of BSA to the immune-reaction solution can stabilize the antibodies and enhance the formation of immunocomplexes.²⁰ The immune-reaction was conducted at room temperature for 30 min.

In CE-LIF analysis, uncoated fused-silica capillary (Yongnian, Hebei, China) was utilized with a dimension of 25 μ m i.d. $\times$ 365 μ m o.d. and effective and total length of 20 and 26 cm, respectively. Prior to first CE-LIF analysis, the capillary was conditioned by 0.1 M NaOH for 4 h, ddH₂O for 10 min, and 1.0 $\times$ TG (pH 8.5, 30 mM Tris, 160 mM glycine) for 4 h, followed by 0.5 $\times$ TG (pH 8.5) electro-equilibrium for 20 min. DNA sample was then electrokinetically injected for 10 s at +20 kV with a high power supply from Tianjin High Voltage Power

Supply Plant (Tianjin, China), and separated by applying constant +20 kV.

The fluorescent species passing detection window were excited by 543.5 nm HeNe laser (1.0 mW, Melles Griot, Irvine, CA) for CE-LIF analysis. The emitted fluorescence was filtered through a 575/15 nm brightline single-band bandpass filter (Semrock, Rochester, NY) and successively amplified to electric current signal using photomultiplier tube (PMT, Hamamatsu Photonics, Japan). The current signal was then converted to voltage signal using a current-to-voltage converter, and finally collected at a frequency of 60 Hz by HW-2000 chromatographic workstation from Qianpu software company (Shanghai, China). Data processing was also conducted on the workstation.

Capillary Electrophoresis (CE)-Single Molecule Fluorescence Imaging (SMFI) Analysis. CE-SMFI analysis was performed on an Olympus IX81 inverted fluorescence microscope in a TIRF (total-internal reflection fluorescence) configuration with PLANPO 60 $\times$ objective (N.A. = 1.42, Olympus, Tokyo, Japan), online coupled with a laboratory-built CE system.²⁶ Note that constant voltage mode was used throughout the experiments. A fused-silica square capillary (Polymicro Technologies, Phoenix, AZ) was fixed on the modified platform with a dimension of 50 μ m i.d., 373 μ m o.d., and total length of 20 cm. Calf thymus DNA (3.5 kb, 4.5 pM) was stained with YOYO-1 iodide (10 nM), and excited by 488 nm laser beam (50 mW, Coherent, Santa Clara, CA). The emitted fluorescence was filtered in order by 506 nm dichroic beam splitter and 525/20 nm brightline single-band bandpass filter (Semrock, Rochester, NY), and collected by electron multiplying charge coupled device (EMCCD) camera (Andor, Ixon^{EM+} DU897, Belfast, Northern Ireland). Exposure time for each frame was set at 50 ms. The images were acquired by Micro Manager 1.3, and stored at 16 bit with a dimension of 256 $\times$ 512 pixels, representing actual scale of 65 μ m $\times$ 130 μ m. Images were processed by ImageJ (National Institutes of Health, free software).³⁰

■ RESULTS AND DISCUSSION

t-ITP Focusing of DNA-Protein Complexes Enhanced by Spontaneously Dissolution of Aerial Carbon Dioxide.

We examined the t-ITP focusing of DNA-protein complexes using sensitive capillary electrophoresis-laser-induced fluorescence (CE-LIF) analysis. The CE-LIF immunoassay of benzo(a)pyrene diol epoxide (BPDE)-dG adducts in genomic DNA^{31,32} was used as an example. BPDE-dG adducts have been exploited as an important biomarker reflecting the internal exposure and cancer risk of carcinogenic polycyclic aromatic hydrocarbons.^{33–35} To perform CE immunoassay of BPDE-dG adducts in genomic DNA, mouse monoclonal antibody 8E11 was chosen as primary antibody (1° Ab) to specifically recognize BPDE-dG adducts, and Alex Flour 546 labeled goat antimouse IgG₁ fragment Fab was chosen as secondary antibody (2° Ab). By this immunoassay format, fluorescently labeled immunocomplexes of BPDE-dG adducts (2° Ab-1° Ab-DNA) can form to be responsive to online coupled LIF detection (see peak 2 in Figure 1).

To achieve t-ITP focusing of the immunocomplexes of BPDE-dG adducts, Tris-glycine buffer (TG buffer, pH 8.5, 0.5 $\times$) and Tris-acetate buffer (TA, pH 7.8, 2 $\times$) were selected as background electrolyte and sample buffer, respectively. In this t-ITP format, acetate anions of high electrophoretic mobility ($\mu_{Ac^-} = -4.27 \times 10^{-4}$ cm² V⁻¹ s⁻¹) in sample buffer and glycine

Figure 1. Enhanced t-ITP focusing of BPDE-dG adduct-antibody complexes using a 10-days or 20-days stored background electrolyte. All the samples were prepared with $2.0\times$ TA buffer (pH 7.8). $0.5\times$ TG (pH 8.5) was used as the background electrolyte. The background electrolyte was freshly prepared (bottom trace), or stored in capped bottle for 10 days (middle trace) or 20 days (top trace) at room temperature. (A) $(\pm)$ -anti-BPDE treated genomic DNA ($5.0\text{ }\mu\text{g/mL}$). (B) DMSO-treated genomic DNA ($5.0\text{ }\mu\text{g/mL}$) as negative control. Peaks 1 and 2 are BPDE-dG adduct- 1°Ab - 2°Ab immunocomplexes and the mixture of unbound 2°Ab and 1°Ab - 2°Ab complexes, respectively.

anions of low electrophoretic mobility ($\mu_{\text{glycine}^-} = -1.9 \times 10^{-5}\text{ cm}^2\text{ V}^{-1}\text{ s}^{-1}$ at pH 8.5) in background electrolyte served as leading and terminating anions, respectively.

First, we tested freshly prepared TG buffer as a background electrolyte. We observed that the immunocomplexes of BPDE-dG adduct (peak 1 in bottom trace, Figure 1A) are reluctantly detectable in the genomic DNA extracted from mouse embryonic stem cells treated by 100 nM $(\pm)$ -anti-BPDE for

2 h. Then, we tested a 10- or 20-days stored-TG buffer (capped but not tightly sealed) as background electrolyte. Surprisingly, the signal of the immunocomplexes of BPDE-dG adducts increases greatly (peak 1 in middle and top traces, Figure 1A), showing the peak height of the immunocomplexes of BPDE-dG adducts increasing from 325 au to 4038 au (arbitrary unit). In contrast, the signal of the immunocomplexes of BPDE-dG adduct is negligible in the genomic DNA extracted from unexposed cells whether the background electrolyte is fresh or stored (Figure 1B).

The above results clearly prove that the known t-ITP format is not effective for the focusing of DNA-antibody complexes. Astonishingly, the 10 days or 20 days stored background electrolyte enhances the t-ITP focusing of DNA-antibody complexes.

As we know, no substances were artificially added after the preparation of the background electrolyte (TG buffer). To our best knowledge, no chemical reaction could take place between inert Tris and glycine in capped TG buffer during storage. On the other hand, only air can slowly penetrate through the capped bottle and dissolve in the buffer. Therefore, there might be an unknown air component changing the property of the TG buffer.

Considering its distribution in air as the fourth highest gas (0.03%) and its tendency to dissolve in alkaline solution,^{36–38} we speculate that aerial carbon dioxide can spontaneously dissolve in the TG buffer (pH 8.5, as background electrolyte) and thus change the physicochemical property of the buffer.

To examine this possibility, we added one piece of dry ice (solid carbon dioxide, $\sim 12.5\text{ g}$) to freshly prepared TG buffer ($0.5\times$, pH 8.5, 1.0 L). After 1 min bubbling of dry ice in TG buffer, we quickly took out 25 mL of TG buffer. The collected buffer (without containing any solid dry ice) was placed at room temperature for 2 h, which might allow the dissolved carbon dioxide to reach chemical equilibrium in the TG buffer. The carbon-dioxide-dissolved TG buffer was used as a background electrolyte and immediately applied for the CE-LIF immunoassay of BPDE-dG adducts.

As we predicted, the signal of the immunocomplexes of BPDE-dG adducts increases with a peak height from 321 au to 1734 au for 1 min dissolution of dry ice (Figure 2). Interesting, the longer dissolution of dry ice (2–20 min) will reduce significantly the pH of the buffer (from pH 8.5 to pH 6.8) and diminish the focusing efficiency. On the other hand, the results also prove that pH change will not contribute to the observed enhancement on t-ITP focusing.

Enhanced Focusing of DNA-Protein Complexes by HCO_3^- -Containing Buffer. In essence, the dissolved aerial carbon dioxide first forms carbonic acid, which eventually dissociates to ionic HCO_3^- and CO_3^{2-} in TG buffer^{36,39} as described by eqs 1–3 below.

The solubility of carbon dioxide is known to be about 1.45 g/L at $25\text{ }^\circ\text{C}$ ($\sim 30\text{ mM}$),^{36,39} and the equilibrium constant for formation of carbonic acid and the $\text{p}K_{\text{a}}$ values of H_2CO_3 and HCO_3^- are also known as those listed in eqs 1–3. Therefore, by assuming that dissolved aerial carbon dioxide is saturated in background electrolyte, we are able to calculate the

Figure 2. Dissolution of dry ice in freshly prepared background electrolyte enhances t-ITP focusing of BPDE-dG adduct-antibody complexes. All the samples were prepared with 2.0× TA buffer (pH 7.8), and 0.5× TG (pH 8.5) was used as the background electrolyte. Peaks 1 and 2 are BPDE-dG adduct-1°Ab-2°Ab immunocomplexes and the mixture of unbound 2°Ab and 1°Ab-2°Ab complexes, respectively.

concentrations of HCO_3^- and CO_3^{2-} using eqs 1–3 and listed equilibrium constants. The concentrations of HCO_3^- and CO_3^{2-} in 0.5× TG at pH 8.5 are estimated as 6.9 and 0.02 mM, respectively. Evidently, HCO_3^- is the major ionic species of carbonic acid, and may be involved in enhancing t-ITP focusing. As a decrease in pH or an increase in acidity, the fraction of HCO_3^- decreased (Figure 2). Correspondingly, the focusing efficiency diminished. This supports a role of HCO_3^- played in the observed focusing.

To test the above hypothesis, we further examined the effect of HCO_3^- on t-ITP focusing using NH_4HCO_3 and Na_2CO_3 . In these two experiments, either NH_4HCO_3 (0–4.0 mM) or Na_2CO_3 (0–2.0 mM) was directly added to the freshly prepared 0.5× TG buffer (pH 8.5). Both NH_4HCO_3 and Na_2CO_3 included in the background electrolyte (0.5× TG buffer, pH 8.5) can improve the signal of the immunocomplexes of BPDE-dG adducts over 10-fold (peak 1, Figure 3). The increase in signal of the immunocomplexes of BPDE-dG adducts is also HCO_3^- -concentration dependent. The enhanced t-ITP signal of immunocomplexes linearly increases from 849 au to 4063 au with an increasing concentration of NH_4HCO_3 from 0.1 to 2.0 mM. The signal of immunocomplexes is kept almost constant with 2.0–4.0 mM NH_4HCO_3 . However, the signal of immunocomplexes decreases after a further increase in the concentration of NH_4HCO_3 (~6.0 mM) (data not shown). Similar enhancement was obtained for Na_2CO_3 from 0.01 to 2.0 mM. Interestingly, even trace ionic HCO_3^- (~0.01 mM) could enhance t-ITP focusing (bottom panel, Figure 3).

Coincidentally, the optimal concentration of HCO_3^- enhancing t-ITP focusing of DNA (2.0–4.0 mM) is comparable to that as estimated for the dissolved aerial carbon dioxide in TG buffer (6.9 mM), confirming the role of the dissolved aerial carbon dioxide in background electrolyte played in the enhancement of t-ITP focusing of BPDE-dG adducts-antibodies complexes (Figure 1).

Taken together, the dissolution of aerial carbon dioxide in background electrolyte indeed enhances the t-ITP focusing of

Figure 3. t-ITP focusing of BPDE-dG adduct-antibody complexes enhanced by NH_4HCO_3 (A) or Na_2CO_3 (B). Either NH_4HCO_3 or Na_2CO_3 was used as an additive in freshly prepared background electrolyte (0.5× TG, pH 8.5). All the samples were prepared with 2.0× TA buffer (pH 7.8). Peaks 1 and 2 are BPDE-dG adduct-1°Ab-2°Ab immunocomplexes and the mixture of unbound 2°Ab and 1°Ab-2°Ab complexes, respectively.

DNA. Considering the pK_a of H_2CO_3 (6.4) and HCO_3^- (10.3), we should point out that both NH_4HCO_3 and Na_2CO_3 share the same major anionic species (HCO_3^-) in the background electrolyte (pH 8.5). In Na_2CO_3 -added buffer (pH 8.5), the major anion is not CO_3^{2-} . Thereby, the above observations further support that the enhanced electrophoresis focusing results from the dissociated form of carbonate HCO_3^- .

Real Time Evolution of Electric Field Revealed by CE-SMFI Analysis. In the t-ITP focusing experiments, the leading ion is acetate from the samples (prepared with 2.0× Tris-acetate buffer), and the terminator is glycine from the background electrolyte (TG buffer) if there is no dissolution of aerial carbon dioxide in the background electrolyte. The samples were electrokinetically injected by applying a voltage of 10 kV for 10 s. The injected volume of acetate anion-containing samples is estimated about 7.6 nL, accounting for 1/17 of the total capillary volume (127.6 nL). Compared with the limited volume of shortly injected acetate anions (expected to be

Figure 4. Profiles of DNA migration velocity (A, C) and electric field strength (B, D) obtained by CE-SMFI analysis. Two t-ITP formats were constructed with freshly prepared buffer of 0.5X TG, pH 8.5 (A, B) or 0.5X TG + 2.0 mM NH_4HCO_3 (C, D) as the background electrolyte. The background electrolyte containing YOYO-1 iodide-stained calf thymus DNA (3.5 kb, ~ 4.5 pM) was electrokinetically filled into the capillary, followed by a pulse injection of 2.0X TA buffer (pH 7.8) by applying a voltage of +1 kV for 10 s. Then, the same background electrolyte but without containing YOYO-1 iodide-stained calf thymus DNA was continuously injected into the capillary. The motion of single DNA molecules was recorded using online coupled SMFI.

leading ions in the tested t-ITP), the HCO_3^- resulting from the dissolution of aerial carbon dioxide in the background electrolyte (0.5X TG buffer) was continuously injected along the CE separation. Therefore, HCO_3^- would become the alternative leading anion due to its high electrophoretic mobility ($\mu_{\text{HCO}_3^-} = -4.61 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$), greatly enhancing t-ITP focusing of immunocomplexes of BPDE-dG adducts.

However, it is not clear how HCO_3^- changes t-ITP format to improve the focusing of DNA in detail. Since any electrophoretic focusing of DNA (including t-ITP and ITP) is dependent on the changes in velocity and the underlying electric field strength, it is useful to monitor in real time the velocity and electric field strength throughout CE separation to the understanding of the relevant focusing mechanism.

Benefiting from our recently developed approach of CE-single molecule fluorescence imaging (SMFI),²⁶ we are able to monitor the real-time motion of DNA throughout the electrophoresis. The resultant velocity profiles of YOYO-1 iodide-stained DNA are shown in Figure 4A,C.

It is known that the velocity (v) is proportional to the electric field strength (E) as shown below.

$$v_{\text{DNA}} = v_{\text{EOF}} + \mu_{\text{DNA}} * E \quad (4)$$

$$v_{\text{EOF}} = \mu_{\text{EOF}} * E_{\text{av}} \quad (5)$$

$$E = (v_{\text{DNA}} - \mu_{\text{DNA}} * E_{\text{av}}) / \mu_{\text{DNA}} \quad (6)$$

Therefore, the E values could be derived with the constant and measurable electrophoretic mobility of DNA (μ_{DNA}) if the velocity of EOF (v_{EOF}) maintains constant.

We measured the velocity and mobility of EOF in the t-ITP format using a neutral fluorescent dye, coumarin 334,^{40,41} following previous protocol.²⁶ It is found that v_{EOF} maintains as a constant through t-ITP-based electrophoresis format (Supporting Information Figures S1 and S2). Due to mass balance, the v_{EOF} is the product of average electric field strength (E_{av}) and the EOF mobility (μ_{EOF}) (see eq 5); thereby, μ_{EOF} was estimated to be $8.10 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ (Supporting Information Figure S2). With the estimated mobility of EOF and the constant electric field strength ($E = 50 \text{ V/cm}$), we further measured the electrophoretic mobility of DNA (μ_{DNA}). It is estimated to be about $-3.88 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$.

With the measured velocity of DNA (v_{DNA}) at each time point and constant v_{EOF} and μ_{DNA} , the electric field strength (E) at each time point could be obtained according to eq 6. Therefore, the E - t curves could be derived from the data of v_{DNA} provided in Figure 4A,C. The final profiles of E - t are shown in Figure 4B,D.

With freshly prepared 0.5X TG buffer (pH 8.5) as the background electrolyte and one pulse injection of TA buffer (10 kV $\times$ 10 s), we observed a local high- E zone (100–125 V/cm) flanking two low- E zones ($\sim 60 \text{ V/cm}$) in Figure 4B. Although the pattern of the electric field distribution as three zones is similar to that for EOF driven-ITP mode ($E \sim 323 \text{ V/}$

cm) described recently,²⁶ the observed zone of high E is narrower ($\sim 1/2$) and shows a lower E value ($\sim 1/3$).

Normally, DNA molecules migrate toward negative end due to the presence of strong EOF in uncoated fused-silica capillary-based electrophoresis,^{35,36} which is used throughout our experiments. However, it is known that DNA molecules can also migrate toward positive end if EOF is suppressed as in the neutrally coated capillary-based electrophoresis. Corresponding to the presence of local high-E zone, the electrophoretic velocity of DNA molecules (toward positive end) locally increases, but the EOF velocity (toward negative end) is maintained constant throughout the capillary due to mass balance.^{29,42} Therefore, the velocity sum of DNA molecules (in the high-E zone) suddenly reduces and even changes direction to move toward positive end (Figure 4A and Supporting Information video 1). The maximum velocity of DNA molecules (toward positive end) is about 88 $\mu\text{m/s}$. In contrast, DNA molecules move toward negative end at a velocity of 170–180 $\mu\text{m/s}$ in the non-high-E zone. Due to the abrupt velocity change, DNA molecules would move toward the LE/TE boundary of t-ITP. When they reach the LE/TE boundary of t-ITP, the velocity of DNA molecules will change again and migrate toward the negative end since the electric field strength (E) returns to a low value (Figure 4B). Therefore, these DNA molecules could be focused at the LE/TE boundary of t-ITP. This explains why we could see a sharp peak of BPDE-dG adducts-antibodies immunocomplexes as shown in Figure 1A (bottom trace).

If we included 2.0 mM NH_4HCO_3 in freshly prepared 0.5 $\times$ TG buffer (pH 8.5), we also observed the three-zone pattern of electric field distribution (Figure 4D). Interestingly, the E value of the high-E zone increases up to 187 V/cm (Figure 4D). In contrast, the E value of the high-E zone is only about 100–125 V/cm if no HCO_3^- ions are included in the background electrolyte (freshly prepared 0.5 $\times$ TG buffer, pH 8.5) (Figure 4B). Consequently, DNA molecules (in the enhanced high-E zone) migrate toward the positive end and the velocity of these molecules increases to 265 $\mu\text{m/s}$ (Figure 4C and Supporting Information video 2). The increased velocity will allow more DNA molecules to reach the LE/TE boundary of t-ITP; therefore, the focusing is greatly enhanced as shown in Figures 1–3. This explains why t-ITP focusing is enhanced by the dissolved HCO_3^- .

On the basis of the enhanced t-ITP focusing of DNA by ionic HCO_3^- , we could detect BPDE-dG adducts ranging from 5.9×10^{-13} mol/L to 2.34×10^{-11} mol/L (data not shown). Furthermore, the addition of NH_4HCO_3 or Na_2CO_3 to the background electrolyte could efficiently and reproducibly enhance t-ITP focusing, accompanying the elimination of the t-ITP fluctuation induced by the uncertain dissolution of carbon dioxide.

In this work, we demonstrate that pure t-ITP focusing is not effective in the presence of EOF. As revealed by real-time CE-SMFI analysis, the observed high-E zone that is critical to any ITP focusing²⁶ is too narrow, and the E value of the high-E zone is not high enough to promote all target ions to move toward the LE/TE boundary.

Interestingly, we show that the dissolution of aerial carbon dioxide in electrophoresis buffer could efficiently improve the t-ITP focusing. The enhancement is related to the main species (HCO_3^-) of dissolved aerial carbon dioxide in weak alkaline buffer. The continuous input of HCO_3^- into the capillary channel can promote the formation of high-E zone with higher

E value, favoring more target ions moving toward and focusing at the LE/TE boundary.

Benefiting from the revealed focusing mechanism, here we propose a new ITP approach to improve the focusing of anions (e.g., DNA, DNA-antibody complexes) using HCO_3^- -containing background electrolyte. The approach improves the focusing efficiency (over 10-fold) and reproducibility.

By the use of real-time CE-SMFI, we gained insights into the dynamic t-ITP focusing process and explored the underlying focusing mechanism. Essentially, the real-time CE-SMFI is a powerful tool for investigation of dynamic electrophoresis processes and for developing new electrophoresis approaches.

CONCLUSIONS

In summary, we demonstrate that the dissolution of aerial carbon dioxide in background electrolyte essentially changes t-ITP format and enhances the focusing of DNA molecules through its ionic species HCO_3^- . Our CE-SMFI study reveals that the presence of HCO_3^- in background electrolyte enhances the formation of the high-E zone, which promotes more DNA molecules to rapidly move backward to and to be efficiently stacked at LE/TE boundary, leading to an effective electrophoresis focusing of DNA molecules.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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Notes

The authors declare no competing financial interest.

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