Published in final edited form as:

Electrophoresis. 2007 October ; 28(20): 3786–3793.

Enhanced pH-Mediated Stacking of Anions for Capillary Electrophoresis Incorporating a Dynamic pH Junction

Stacy D. Arnett and Craig E. Lunte*

Ralph N. Adams Institute of Bioanalytical Chemistry, Department of Chemistry, University of Kansas, Lawrence, KS 66047

Abstract

A technique has been developed to enhance analyte focusing for capillary electrophoresis for the analysis of physiological samples. High-ionic strength samples are titrated to low-ionic strength on-line using pH-mediated sample stacking in conjunction with a dynamic pH junction. This method concentrates analytes by reducing their electrophoretic mobility during field-amplification. Parameters responsible for enhanced focusing were investigated, and an enhanced pH-mediated stacking method was optimized for anionic nucleosides. The process results in ultra-narrow peak widths, for example, 0.28 s for thymidine with a 10-minute analysis time. Peak width and resolution with the enhanced stacking method were also compared to normal base stacking and electrokinetic injection. With this technique, mass-loading capacity can be increased without degradation in peak shape and resolution is dramatically improved.

Keywords

pH-mediated stacking; dynamic pH junction; online sample preconcentration

1. INTRODUCTION

Capillary electrophoresis (CE) has become a widely used analytical technique for the analysis of biological samples, especially for compounds that are difficult to resolve by reverse-phase liquid chromatography (LC). CE is an attractive separation technique because analyses can be performed on much smaller sample volumes at higher speed, lower cost, and with much less solvent consumption than conventional LC. Biological samples with high-ionic strength matrices such as plasma and microdialysate, however, can be problematic for CE because of significant band broadening during the separation. Sample “destacking” can occur due to the lower field strength in the high-conductivity sample zone, resulting in poor peak efficiency and reduced sensitivity. This can be especially detrimental when using photometric detection since the small capillary dimensions already place a limitation on concentration sensitivity. Improving sensitivity for optical detection methods has been the focus of many investigations [1–4]. The most common method to reverse this effect is to perform a dilution or extraction of the sample into a low-ionic strength matrix in order to increase the field strength in the sample zone relative to the background electrolyte (BGE), and is generally referred to as field-amplified sample stacking [5–7]. Field amplification also increases mass loading since large volume samples can be preconcentrated on-capillary, thus enhancing sensitivity [8–10]. Sample preconcentration and stacking techniques have recently been reviewed by Breadmore [11].

*Correspondence: Craig E. Lunte, University of Kansas, Multidisciplinary Research Building, 2030 Becker Drive, Lawrence, KS 66047-1620, Email clunte@ku.edu, Fax (785) 864-5396.

In order to achieve field-amplified stacking of analytes in high-ionic strength samples without the need for a dilution or extraction step, we have developed a technique termed pH-mediated stacking, which allows the on-line titration of a high ionic strength sample matrix to low ionic strength. It can be performed for either cationic or anionic analytes by “acid stacking” [12] or “base stacking” [13,14], respectively. The focus of this research involves the use of base stacking, where the electroosmotic flow (EOF) is reversed and the separation is performed with reverse polarity. The BGE consists of the salt of a weak base, such as the ammonium ion. Sample injection is performed electrokinetically, during which sample cations (predominately Na^+ in physiological samples) are displaced by “titrable” BGE cations. Sample injection is immediately followed by an electrokinetic injection of basic solution, during which hydroxide ions titrate BGE cations in the sample band and create a zone of low conductivity. Field amplification then occurs in this titrated zone, and anionic analytes will be concentrated at the interface of the low-conductivity titrated zone and the high-conductivity BGE.

Here we report a significant enhancement of the base stacking technique that results in the achievement of peak widths for CE near the peak width predicted by diffusion only. In this new technique, a discontinuous BGE of varying pH is employed (Figure 1). A short plug of BGE, with a lower pH than the separation BGE, is injected hydrodynamically prior to sample injection. The lower-pH BGE ahead of the sample zone serves to reduce the electrophoretic mobility of the analytes by reducing the analyte charge state and creating a dynamic pH junction [3,15–17]. Sample injection and subsequent hydroxide injection occur as previously described for normal base stacking. In addition to encountering a field strength interface, the analytes now also experience a loss in mobility, which enhances the sample stacking effect. Before reaching the detection window, analytes are allowed minimal time to regain charge and separate in the higher-pH BGE. The ability to concentrate sample bands to very narrow peak widths and high resolution greatly increases sensitivity and mass loading capacity, which can greatly reduce concentration limits of detection for CE.

Multi-million plate peak efficiencies have been reported for CE previously for large biomolecules with very small diffusion coefficients such as DNA [18] for peptides with very high separation voltages [19] or for amino acids with many hours exposure to the separation field [20]. The pH-mediated stacking method reported here requires no specialized equipment and achieves high-efficiency separations in typical CE run times. Although nucleosides were chosen to demonstrate the enhanced base stacking method, it can be universally applied to any weakly acidic analyte that can be titrated to achieve reduced mobility. Because this is a complex stacking mechanism involving field amplification and velocity-difference induced focusing, we have chosen to use peak width rather than plate number as an efficiency parameter.

In this report, the development of an enhanced pH-mediated stacking method incorporating a dynamic pH junction is described, beginning with the initial observation of greatly reduced peak widths under specific base stacking conditions. Factors responsible for reduced peak widths were determined and then incorporated into a general method for achieving narrow peak widths and enhanced resolution for anions.

2. MATERIALS AND METHODS

Materials

8-hydroxy-2'-deoxyguanosine (8OHdG), 2'-deoxyguanosine (dG), thymidine (dT), hydroxybenzoic acid (HBA), glutathione disulfide (GSSG), naproxen, corticosterone, imidazole hydrochloride, tris(hydroxymethyl)aminomethane hydrochloride (Tris), ammonium chloride, methylamine hydrochloride, and tetradecyltrimethylammonium bromide (TTAB) were purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade or better

and used as received. All solutions were prepared in distilled, deionized water (Labconco, Kansas City, MO) and were filtered through a 0.22- μ m pore size membrane filter prior to use.

Instrumentation and General Procedures

Fused-silica capillary (50 μ m i.d.) was obtained from Polymicro Technologies (Phoenix, AZ) and cut to a total length of 60 cm (45 cm to detection window). New capillaries were pretreated by rinsing at 20 psi with methanol for 10 minutes, 0.1 M HCl for 5 minutes, water for 2 minutes, 1.0 M NaOH for 10 minutes, water for 2 minutes, and BGE for 5 minutes. Before each injection, capillaries were rinsed for 2 minutes each with 0.1 M NaOH, water, and BGE at 20 psi. BGEs were prepared at a concentration of 100 mM with 0.5 mM TTAB added to reverse the EOF. pH was adjusted with 5 M sodium hydroxide solution. A high voltage power supply (Spellman High Voltage Electronics Corp., Hauppauge, NY) was used to drive electrophoresis at -12 kV (negative polarity mode). Under these conditions, the electromigration of anions and the EOF are towards the detector. The cathodic capillary reservoir (inlet) and electrical connections were isolated in a Plexiglas safety box fitted with an interlock. Experiments were performed at ambient temperature. Detection was performed with a Spectra-Physics (San Jose, CA) SpectraSYSTEM UV1000 UV-vis detector (with a flow cell modified for CE) at 254 nm. Data collection was performed at 50 Hz with a serial port-powered A/D converter and Chrom&Spec 1.5x software (Ampersand International, Inc., Beachwood, OH). Wavemetrics Igor Pro 3.12 and Microcal Origin 6.0 were used for data analysis. A 1 mM stock solution of each analyte was prepared in water and standard solutions were diluted from stock daily in either BGE or Ringer's solution (145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂) as noted.

Peak Widths with Normal Base Stacking as a Function of BGE / pH

BGE pH was adjusted in small increments within or slightly below the buffering range of each buffer salt. 8OHdG standard (50 μ M) in Ringer's solution was injected at -12 kV for 30 s in each BGE at varying pH, immediately followed by a 0.1 M NaOH injection at the same voltage. The minimum length of the NaOH injection required to stack 8OHdG in each BGE type has been determined previously [14]. For a 30 s sample injection in imidazole, Tris, ammonium, and methylamine BGE, the length of NaOH injection was 15 s, 15 s, 20 s, and 40 s, respectively.

Analyte Mobility as a Function of pH

BGE pH was adjusted in increments of 0.5. Imidazole BGE was used for pH 7.0 and 7.5, Tris for pH 8.0 and 8.5, and ammonium for pH 9.0 and 9.5. Analyte standards (100 μ M HBA, GSSG, naproxen, 8OHdG, dG, and dT) were prepared in each BGE, at both pH values. Corticosterone (100 μ M) was added to each standard as a neutral marker. Standards were injected for 5 s at 0.5 psi in the appropriate BGE at each pH increment. For these experiments, separation was performed at -10 kV.

Reduced Peak Widths for Other Anions by Manipulating pH

BGEs were prepared at pH increments of 0.1. Tris was used for pH 8.5 - 7.5 and imidazole was used for pH 7.4 - 6.5. A standard mixture containing 50 μ M of each analyte (naproxen, 8OHdG, dG, and dT) was prepared in Ringer's solution. This standard was injected at -12 kV for 30 s for each BGE, followed by a 15 s injection of 0.1 M NaOH at the same voltage.

Enhanced pH-Mediated Stacking with a dynamic pH junction of Anions

Before an enhanced base stacking method was optimized, the minimum duration of NaOH injection required to stack a 20 s injection of dG and dT standards in Ringer's was determined with normal base stacking as described earlier. The enhanced base stacking procedure is outlined in Figure 1. For enhanced base stacking with a dynamic pH junction, the pressure and length of the hydrodynamic injection of lower-pH BGE was optimized experimentally for Tris

and imidazole BGEs at various pH values to obtain the highest efficiency. Injection times were typically varied by 5 s intervals and pressure was varied at 5 psi intervals initially, and then varied at smaller increments until optimal peak efficiencies were reached with a particular BGE type and pH. For peak width calculations, migration time (t_m) was measured from a CE separation where the analyte was dissolved in methylamine BGE at pH 10.5 (used as the high-pH BGE in all experiments). Actual peak widths were also determined from these CE experiments for comparison. The pH at which the mobility (μ) of each analyte approaches zero with normal CE was determined by varying the pH in 0.5 increments in methylamine, Tris, or imidazole BGE within the buffering range of each salt. For enhanced stacking, the high-pH BGE was methylamine at pH 10.5 and the low-pH BGE was imidazole. Samples analyzed with a base stacking technique were dissolved in Ringer's solution to mimic extracellular fluid pH and ionic strength. Analytes dissolved in Ringer's solution cannot be detected with normal CE due to sample destacking, and therefore were dissolved in BGE for comparison. Theoretical peak widths based on longitudinal diffusion only were calculated using equation 1

$$W_{\frac{1}{2},\text{diff}} = 2.35 \sqrt{2D \cdot t_m} \quad (1)$$

where $W_{\frac{1}{2},\text{diff}}$ is the width at half the peak height, D is the diffusion coefficient for small molecules, and t_m is the migration time (s) in normal CE. Peak width calculations were made using a diffusion coefficient range of 1×10^{-6} to $5 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1}$, typical for small organic molecules [21].

3. RESULTS AND DISCUSSION

Reduced Peak Widths as a Function of BGE / pH

Dramatically narrowed peak widths were first observed while developing a CE separation for 8OHdG in microdialysis samples with base stacking. In order to determine the parameter(s) responsible for this considerable peak narrowing, the peak width for 8OHdG in the four amine BGEs at varying pH values was measured (Figure 2). The ranges of experimental pH values were chosen so as not to greatly exceed the buffering range of the individual BGE. The narrowest peak width for 8OHdG was achieved between ~ pH 6.5 – 7.5 in Tris and imidazole BGEs, and appeared to be a function of pH. The local maxima occurred slightly below the pK_a of imidazole and Tris, but this trend was not evident in the other two BGEs. The pK_a of 8OHdG has been estimated as ~ 8 [22]. This is approximately one pH unit above the pH at which a marked decrease in peak width occurred in Tris and imidazole BGE. Since analyte pK_a determines analyte charge (and thus mobility in CE), analyte mobility was suspected to be a major factor in achieving ultra-narrow peak widths with base stacking.

8OHdG was subsequently stacked in imidazole BGE (pH 7.0) at increasing sample injection times while maintaining the injection ratio, to demonstrate the enhanced sensitivity achieved with ultra-narrow peak widths (data not shown). There was a linear relationship between peak height as a function of injection time and 8OHdG could be injected for at least 6 minutes without deterioration of peak shape. For a 6 minute injection, the detection limit for 8OHdG was 100 nM with UV detection. This detection limit is 3 times lower than previously reported with base-stacking with UV detection [13].

Analyte Mobility as a Function of pH

To further explore the hypothesis that peak widths using base stacking are dependent on analyte mobility, the mobility of five analytes was measured at varying pH in amine BGEs. Figure 3 shows a plot of anion mobility as a function of pH. As the pH of the BGE was lowered, the mobility of the anions decreased. The mobility of HBA and GSSG did not approach zero in the pH range studied. Naproxen mobility did not change significantly, but a tailing peak shape indicated that it may interact with TTAB and/or the capillary wall. Table 1 lists the pH value

where the mobility of each analyte approached zero, determined from the point where only partial resolution of each analyte from the neutral marker was observed. The mobility of 8OHdG approached zero around pH 7.5, while the mobility of dG and dT approached zero around pH 8.5.

Reduced Peak Widths for Other Anions with Base Stacking

Naproxen, dG, and dT were chosen to determine whether the ultra-narrow peak widths achieved with 8OHdG could also be achieved for other anions based on the mobility data in the amine BGEs (Figure 3). HBA and GSSG were excluded since their pK_a s (4.6 and 3.5 (pK_{a2}), respectively) were far below the buffering range of imidazole (the amine BGE with the lowest pK_a used in this study). Reported pK_a s for naproxen, dG, and dT are 4.9, 9.24 [23], and 9.8, respectively. An attempt was made to achieve ultra-narrow peak widths for these analytes by manipulating the BGE pH in normal base stacking. Peak narrowing should occur as the pH of the BGE reaches the point where analyte mobility approaches zero. It should be noted that pH-mediated stacking was only effective for cationic or anionic analytes, which implies that analytes must have mobilities greater than zero for enhanced stacking to occur with the normal pH-mediated stacking method. Base stacking was performed in each amine BGE at decreasing pH values, so as not to extend beyond the buffering capacity of the individual BGE. Electropherograms at selected pH values are shown in Figure 4. Naproxen, 8OHdG, dG, and dT were all successfully stacked with sub-second peak widths at specific pH values. Table 1 lists the BGE pH value with normal base stacking where sub-second peak widths occurred for each analyte as the pH was lowered. These pH values are approximately the pH values where the mobility of each analyte approaches zero in normal CE (Table 1). However, as the electropherograms in Figure 4 illustrate, co-migration occurred as the pH was decreased to facilitate enhanced stacking of each anion. Peak areas confirmed that as each anion successively underwent enhanced stacking, it co-migrated with the previous peak(s). This is indicative of the presence of an interface between regions of differing ionic strength, where analytes are known to stack [13]. Therefore, co-migration is most likely to occur due to the combination of an ionic strength interface and similar low mobilities of analytes as the pH is decreased. Lowering the pH of the BGE used in normal base stacking, therefore, triggers dramatic peak-narrowing but does not provide the resolution necessary for the separation.

Enhanced pH-Mediated Stacking of Anions with a Dynamic pH Junction

In order to achieve resolution of anionic analytes but also maintain ultra-narrow peak widths, the base stacking method was modified with a dynamic pH junction. An enhanced base stacking procedure (Figure 1) was optimized for the separation of 8OHdG, dG, and dT. Naproxen was not included since it had a significant interaction with the capillary wall that led to considerable peak tailing. To optimize enhanced base stacking conditions for the three nucleosides chosen for this study, the ratio of sample injection length to hydroxide injection length was first optimized with normal base stacking in the higher-pH BGE. To create an enhanced stacking method, a second titrable BGE was chosen to create a lower-pH zone ahead of the sample zone. The pH must be sufficiently low so analyte mobility approaches zero as it enters this zone. The lower-pH BGE plug must also be large enough so analytes do not regain their charge state too soon before passing the detection window and undergo band-broadening due to longitudinal diffusion in the absence of the pH/ionic-strength interface. Methylamine BGE at pH 10.7 was chosen as the higher-pH BGE. When analytes encounter this BGE zone, all three analytes should be negatively charged based on their pK_a s and mobility data.

Table 1 compares analyte pK_a , the pH in normal CE where μ approached zero, the pH where enhanced stacking occurred with normal stacking (without resolution), and the pH of the lower-pH BGE required to achieve resolution with enhanced stacking. When the BGE pH is lower than the pK_a s of the nucleosides, they will have almost no negative charge and thus almost no

electrophoretic mobility. Enhanced stacking will occur during normal stacking when the pH of the BGE is near the pH value where the analyte mobility reaches zero in CE. Although enhanced stacking can occur during normal stacking, peak resolution is not maintained. To achieve enhanced stacking with resolution, the pH of the lower-pH BGE injected before the sample must be significantly lower than the pH where analyte mobility reaches zero in CE. Since the lower-pH plug will mix with the higher-pH BGE in the capillary, the lower-pH BGE must be more acidic than that required for the analyte mobility to reach zero. Optimization of the pH of the lower-pH BGE, rather than injection volume, had the greatest impact on peak width. For a 12 s, 10 psi injection of the lower-pH BGE, sub-second peak widths were achieved for each analyte when the pH was approximately two pH units below that where analyte mobility reached zero with normal CE. Longer injections at higher pH did not result in the same peak widths. Figure 5 shows an electropherogram before the conditions were fully optimized. In this example, the pH of the low-pH BGE was 6.0. The peak width for dG and dT was less than 0.5 s, while the peak width for 8OHdG was 2.6 s. These were the narrowest peak widths that could be obtained with any length of injection using pH 6.0 BGE. Figure 6 shows an electropherogram under completely optimized conditions with the pH of the low-pH BGE at 4.8. Subsecond peak widths were achieved for all three nucleosides. (As can be inferred from the analyte peak widths and migration times, a traditional calculation of peak efficiencies would yield 3.8, 13.5, and 14.0 million plates for 8OHdG, dG, and dT, respectively.)

Figure 7 compares electropherograms obtained with the enhanced stacking and normal base stacking techniques to an electropherogram obtained by normal CE. A comparison of peak widths, resolution, and signal-to-noise obtained with all three techniques is made in Table 2. Normal base stacking produced peak widths for high-ionic strength samples in the range of 1.7 – 1.9 s (as compared to 5.2 – 5.5 with normal CE for sample in BGE). Using the enhanced base stacking technique peak widths in the range of 0.2 – 0.6 s were achieved for 8OHdG, dG, and dT. This is a decrease of approximately 5-fold compared to normal base stacking, and 20-fold compared to CE. Peak widths of under 0.3 s for dT and dG were achieved under these conditions. This is 15 to 30 % of the peak widths obtained with normal base stacking and 5 to 10 % of peak widths with normal CE. The signal-to-noise ratio for dG with enhanced stacking was 24 times greater than with CE, and 8 times greater than with normal base stacking.

For normal CE experiments, sample could only be injected for 5 s at –12 kV before the peak shape and resolution was degraded. With the base stacking techniques, a 20 s injection of sample did not begin to affect peak shape or resolution. Although the sample used with the base stacking techniques was of higher ionic strength than the BGE and the injection length was four times longer, resolution of the three analytes was greater than with CE. There is a dramatic increase in resolution between enhanced base stacking and normal base stacking, which will allow for even greater injection lengths before the column is overloaded.

4. CONCLUDING REMARKS

We have described a method for the online preconcentration of analytes in high-ionic strength matrices that results in sub-second peak widths with high resolution. The ability to concentrate analytes into such narrow bands results in a dramatic increase in resolution as well as sensitivity. For base stacking, sample injection length is mostly limited by column length, but can be increased indefinitely as long as the ratio of sample injection to hydroxide injection remains constant. For untreated biological samples, the limiting factor for sample injection length will ultimately be whether the concentration of matrix components overwhelms peak capacity. We routinely use electrokinetic sample injection lengths of 60 s for microdialysis samples without peak broadening or loss of resolution.

The information gained from this study can be used to develop a general scheme for rapid method optimization. When analyzing a sample in Ringer's solution or microdialysate, no dilution of the sample to lower ionic strength is necessary. A BGE concentration of 100 mM should be used, using a low pH BGE (< 5) plug before sample injection and a high pH separation BGE (> 10). The injection ratio (sample injection length / NaOH injection length) for the analyte with the lowest mobility should first be determined with normal base stacking. The injection time and pressure for the low-pH BGE should then be optimized to achieve enhanced base stacking using the injection ratio determined for normal base stacking.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health grant R01EB00247. SDA acknowledges the support of the National Cancer Institute from training grant T32CA09242.

References

1. Mazereeuw M, Tjaden UR, Reinhoud N. J. Chromatogr. Sci 1995;33:686–697.
2. Quirino JP, Terabe S. Science 1998;282:465–468. [PubMed: 9774271]
3. Britz-McKibbin P, Wong J, Chen DY. J. Chromatogr. A 1999;853:535–540. [PubMed: 10486763]
4. Cao C-X, He Y-Z, Li M, Qian Y-T, Gao M-F, Ge L-H, Zhou S-L, Yang L, Qu Q-S. Anal. Chem 2002;74:4167–4174. [PubMed: 12199589]
5. Mikkers FEP, Everaerts FM, Verheggen TP. J. Chromatogr 1979;169:1–10. [PubMed: 541389]
6. Chien R-L, Burgi DS. Anal. Chem 1992;64:1046–1050.
7. Swartz ME, Merion M. J. Chromatogr 1993;632:209–213.
8. Maciá A, Borrull F, Aguilar CA. Electrophoresis 2004;25:428–436. [PubMed: 14760634]
9. Xu Y, Qin WD, Lau YH, Li SFY. Electrophoresis 2005;26:3507–3517. [PubMed: 16100745]
10. Hsieh MM, Chang HT. Electrophoresis 2005;26:187–195. [PubMed: 15624169]
11. Breadmore MC. Electrophoresis 2007;28:254–281. [PubMed: 17149782]
12. Hadwiger ME, Torchia SR, Park S, Biggin ME, Lunte CE. J. Chromatogr. B 1996;681:241–249.
13. Zhao Y, Lunte CE. Anal. Chem 1999;71:3985–3991. [PubMed: 10500485]
14. Arnett SD, Lunte CE. Electrophoresis 2003;24:1745–1752. [PubMed: 12783451]
15. Britz-McKibbin P, Chen DDY. Anal. Chem 2000;72:1242–1252. [PubMed: 10740866]
16. Britz-McKibbin P, Bebault GM, Chen DDY. Anal. Chem 2000;72:1729–1735. [PubMed: 10784134]
17. Kim JB, Britz-McKibbin P, Hirokawa T, Terabe S. Anal. Chem 2003;75:3986–3993. [PubMed: 14632109]
18. Guttman A, Cohen AS, Heiger DN, Karger BL. Anal. Chem 1990;62:137–141.
19. Hutterer KM, Jorgenson JW. Anal. Chem 1999;71:1293–1297.
20. Culbertson CT, Jorgenson JW. Anal. Chem 1994;66:955–962.
21. Mills, R.; Lobo, VMM. Self Diffusion in Electrolyte Solutions. Amsterdam: Elsevier; 1989.
22. Culp SJ, Cho BP, Kadlubar FF, Evans FE. Chem. Res. Toxicol 1989;2:416–422. [PubMed: 2519731]
23. Sigel H, Song B, Oswald G, Lippert B. Chem. Eur. J 1998;4:1053–1060.

Abbreviations

8OHdG, 8-hydroxy-2'-deoxyguanosine; dG, 2'-deoxyguanosine; dT, thymidine; HBA, hydroxybenzoic acid; GSSG, glutathione disulfide; TTAB, tetradecyltrimethylammonium bromide.

Figure 1.

Enhanced pH-mediated stacking of anions (base stacking) with a dynamic pH junction. Step 1: A small plug (shaded gray) of lower-pH background electrolyte (BGE) consisting of a weak base is injected hydrodynamically into the capillary filled with higher-pH BGE also consisting of a weak base. Step 2: High-ionic strength sample is injected electrokinetically and titratable BGE cations migrating toward the cathode replace untitratable sample cations (such as Na^+) in the sample zone. Step 3: Basic solution is electrokinetically injected and hydroxide ions titrate BGE cations to form a low conductivity zone in which analyte velocity increases under the higher field strength, when analytes encounter both the high-ionic strength BGE zone and the lower-pH zone, they are concentrated at the interface due to both the lower field strength and decreased charge state. Step 4: The higher-pH BGE vial is replaced and the titrated zone ceases to grow, as analytes exit the low pH, low conductivity zone, they regain charge state and can be separated in the BGE based on their electrophoretic mobilities before reaching the detection window.

Figure 2.

Peak width of 8OHdG as a function of pH / BGE. Conditions: 30 s injection of 50 μ M 8OHdG in Ringer's followed by 0.1 M NaOH at the minimum injection length required to stack 8OHdG imidazole, 15 s, Tris, 15 s, NH_4OH , 20 s, methylamine, 40 s, 100 mM BGE with 0.5 mM TTAB, 50 μ m i.d. capillary $\times$ 60 (45) cm, separation and injection at -12 kV, $n = 5$. Symbols: $\blacktriangle$ imidazole, pK_a 6.9; $\blacksquare$ Tris, pK_a 8.1; $\bullet$ Ammonium, pK_a 9.0; $\star$ Methylamine, pK_a 10.7.

Figure 3.

Analyte mobility as a function of pH. Conditions: 100 mM BGE with 0.5 mM TTAB (imidazole for pH 7.0 and 7.5, Tris for pH 8.0 and 8.5, ammonium for pH 9.0 and 9.5), 100 μ M of each analyte in sample mixture in BGE, 50 μ m i.d. capillary $\times$ 60 (50) cm, separation at -10 kV, hydrodynamic injection at 0.5 psi for 5 s, $n = 3$. Symbols: ■ HBA; ● GSSG; ▲ Nap; ▼ 8OHdG; ★ dG; ◆ dT.

Figure 4.

Peak narrowing for other anions by manipulating pH. Peak identities are: (1) naproxen, (2) 8OHdG, (3) dG, and (4) dT. Conditions: 30 s electrokinetic injection of mixture of 50 μ M of each analyte in Ringer's followed by a 15 s electrokinetic injection of 0.1 M NaOH, 100 mM Tris (pH 8.4, 8.2, 8.0, and 7.6) or imidazole (pH 7.4 and 6.5) with 0.5 mM TTAB, 50 μ m i.d. capillary $\times$ 60 (45) cm, separation and injection at -12 kV. Peak area values appear next to peaks.

Figure 5.

Enhanced base stacking of three nucleosides under partially optimized conditions. Conditions: 20 s hydrodynamic injection of low-pH BGE (100 mM imidazole, 0.5 mM TTAB, pH 6.0) at 10 psi, followed by sample in Ringer's solution injected for 20 s at -12 kV, followed by 30 s injection of 0.1 N NaOH at -12 kV, high-pH BGE was 100 mM methylamine, 0.5 mM TTAB, pH 10.7, separation was performed at -12 kV with a 50 μ m i.d. capillary $\times$ 60 cm (45 cm to detection window), UV detection was performed at 254 nm.

Figure 6. Enhanced base stacking of three nucleosides. Conditions: 12 s hydrodynamic injection of low-pH BGE (100 mM imidazole, 0.5 mM TTAB, pH 4.8) at 10 psi, all other conditions same as in Figure 5.

Figure 7.

Capillary electrophoresis separation of three nucleosides with enhanced base stacking, with normal base stacking, and without base stacking. A: enhanced base stacking, 12 s hydrodynamic injection of low-pH BGE (100 mM imidazole, 0.5 mM TTAB, pH 4.8) at 10 psi, followed by sample in Ringer's solution injected for 20 s at -12 kV, followed by 30 s injection of 0.1 N NaOH at -12 kV, $W_{1/2, dG} = 0.28$ s. B: normal base stacking, sample in Ringer's solution injected for 20 s at -12 kV, followed by 30 s injection of 0.1 N NaOH at -12 kV, $W_{1/2, dG} = 1.32$ s. C: without base stacking, sample in BGE injected for 5 s at -12 kV, $W_{1/2, dG} = 5.52$ s. High-pH BGE in all experiments was 100 mM methylamine, 0.5 mM TTAB, pH 10.7. Separation was performed at -12 kV with a 50 μ m i.d. capillary $\times$ 60 cm (45 cm to detection window), UV detection was performed at 254 nm.

Table 1

Comparison of analyte pK_as, pH values where analyte mobility approaches zero in normal CZE, pH values required to achieve ultra-narrow peak widths with normal base stacking, and the pH of the lower-pH BGE required to achieve ultra-narrow peak widths, and peak resolution with enhanced base stacking

Analyte	pK _a	CZE ^a	Normal Stacking ^a	Enhanced Stacking ^b
		$\mu \rightarrow 0$	$W_{1/2,act} \rightarrow W_{1/2,diff}$ (no resolution)	$W_{1/2,act} \rightarrow W_{1/2,diff}$ (with resolution)
8OHdG	~ 8 ¹²	7.5	7.4	4.8
dG	9.24 ¹³	8.5	8.0	6.0
dT	9.8	8.5	8.2	6.0

^a Refers to pH of separation BGE

^b Refers to lower-pH BGE zone ahead of sample zone, with separation BGE at higher pH

Table 2

Comparison of experimental peak widths ($W_{1/2,act}$), theoretical peak widths based on diffusion only ($W_{1/2,diff}$), peak resolution (R), and signal-to-noise ratio (S/N) for the separation of nucleosides using capillary electrophoresis, $n = 5$. Relative standard deviations are in parentheses. Conditions are the same as in Figure 7. Theoretical peak width using equation 1.

Parameter	Enhanced Stacking (<i>Actual</i>)	Normal Stacking (<i>Actual</i>)	CZE (<i>Actual</i>)	CZE (<i>Theoretical</i>)
$W_{1/2}$, 8OHdG (s)	0.60 (12)	1.86 (12)	5.23 (5.5)	0.15 – 0.21
$W_{1/2}$, dG (s)	0.29 (6.9)	1.80 (11)	5.42 (5.0)	0.15 – 0.21
$W_{1/2}$, dT (s)	0.28 (3.6)	1.74 (10)	5.43 (4.8)	0.15 – 0.22
R , 8OHdG-dG	8.3 (24)	1.8 (5.6)	1.4 (14)	-
R , dG-dT	9.4 (13)	1.9 (5.3)	1.8 (5.6)	-
S/N , dG	1570 (17)	550 (5)	65 (2)	-