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Comparative study on sample stacking by moving reaction boundary formed with weak acid and weak or strong base in capillary electrophoresis: II. Experiments

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ABSTRACT

To demonstrate the theoretic method on the stacking of zwitterion with moving reaction boundary (MRB) in the accompanying paper, the relevant experiments were performed. The experimental results quantitatively show that (1) MRB velocity, including the comparisons between MRB and zwitterionic velocities, possesses key importance to the design of MRB stacking; (2) a much long front alkaline plug without sample should be injected before the sample injection for a complete stacking of zwitterion if sample buffer is prepared with strong base, conversely no such plug is needed if using a weak base as the sample buffer with proper concentration and pH value; (3) the presence of salt in MRB system holds dramatic effect on the MRB stacking if sample solution is a strong base, but has no effect if a weak alkali is used as sample solution; (4) all of the experiments of this paper, including the previous work, quantitatively manifest the theory and predictions shown in the accompanying paper. In addition, the so-called derivative MRB-induced re-stacking and transient FASI-induced re-stacking were also observed during the experiments, and the relevant mechanisms were briefly demonstrated with the results. The theory and its calculation procedures developed in the accompanying paper can be well used for the predictions to the MRB stacking of zwitterion in CE.

1. Introduction

In the accompanying paper [1], the theoretical investigations were performed for the conditions of zwitterionic stacking by moving reaction boundary (MRB) formed with weak acidic buffer and strong/weak alkali in capillary electrophoresis (CE). Numerous significant results were drawn from the theoretical studies [1]. At first, the velocity of MRB, including the comparison of MRB and zwitterion velocities, holds a key importance to the design of MRB stacking of zwitterion. The velocity of boundary should be generally optimized in accordance with the approximate optimized conditions: $0 < V^{\alpha\beta}_{\text{weak}}(\text{or } |V^{\alpha\beta}_{\text{w-s}}|) \leq \bar{V}^{\alpha}_{z+}(\text{or } |\bar{V}^{\beta}_{z-}|)$ ($V^{\alpha\beta}$ is the velocity of MRB, the subscripts of “weak” and “w–s” indicate the weak acid–base and the weak acid–strong base systems of MRB respectively; $\bar{V}^{\alpha}_{z+}$ is the constituent velocity of zwitterion in phase α (usually acidic buffer); $\bar{V}^{\beta}_{z-}$ presents the constituent velocity of zwitterion in phase β (*viz.*, strong alkali herein)).

Secondly, an enough length of strong alkaline plug without zwitterion analyte(s) should be injected before the sample injection for the complete stacking of zwitterion sample, if a strong alkali is used as sample solution. Conversely no such front alkaline plug is needed for the complete stacking of zwitterion if a weak alkali with enough high concentration and proper pH value is used for the preparation of sample matrix of zwitterion. Thirdly, the presence of salt in a sample matrix holds almost no obvious effect on the MRB-based stacking of zwitterion if MRB is created with weak electrolytic systems, e.g., formic buffer and sodium formate used in Refs. [2–4] and acetic buffer and ammonia studied in Ref. [1] and herein. Whereas, the existence of salt has a dramatic effect on the stacking for a strong electrolyte (e.g., NaOH) system, and the dramatic effect further leads the instability of whole MRB system.

Evidently, a weak alkali is of much superiority to a strong alkali. However, there are still no systemic experiments demonstrating the validity of the theory of MRB-based sample stacking, the theoretical computations, and the relevant conclusions achieved in the accompanying paper [1].

Therefore, the main purpose of this work is to prove the validity of theoretical results mentioned above. The experimental method of MRB stacking of zwitterion was designed to perform the systemic experiments on the MRB stacking, compare the experimental results with the theoretical calculations and quantitatively test the

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Table 1
The comparisons between the theoretical predictions and experiments of the stacking of His and Trp by MRB with different boundary velocity in CE.

No.	System	Velocity (m/s)[a]	Prediction[a]	Experiment
1	40 mM pH 3.50 acetic buffer (+, α)||0.4 mM NaOH + 20 mM NaCl (−,β)	$V_{w\text{-}s} = 1.18 \times 10^{-2}$	With Eq. (14b) in Ref. [1]	
		$V_{Trp} = 1.05 \times 10^{-4}$	Poor in the back part of P_{Trp}	Agrees with Fig. 2
		$V_{His} = 2.23 \times 10^{-3}$	Poor in the back part of P_{His}	Agrees with Fig. 5A
2	40 mM pH 3.50 acetic buffer (+,α)||2.0 mM NaOH + 20 mM NaCl (−, β)	$V_{w\text{-}s} = 2.61 \times 10^{-3}$	With Eq. (14b) in Ref. [1]	
		$V_{Trp} = 1.05 \times 10^{-4}$	Poor in the back part of P_{Trp}	Agrees with Fig. 4A
		$V_{His} = 2.23 \times 10^{-3}$	Fair in the back part of P_{His}	Agrees with Fig. 5B
3	40 mM pH 3.50 acetic buffer (+,α)||6.0 mM NaOH + 20 mM NaCl (−,β)	$V_{w\text{-}s} = 3.43 \times 10^{-4}$	With Eq. (14b) in Ref. [1]	
		$V_{Trp} = 1.05 \times 10^{-4}$	Fair in the back part of P_{Trp}	Agrees with Fig. 4B
		$V_{His} = 2.23 \times 10^{-3}$	Good in the back part of P_{His}	Agrees with Fig. 5C
4	40 mM pH 3.50 acetic buffer (+,α)||10 mM NaOH + 20 mM NaCl (−,β)	$V_{w\text{-}s} = -0.04 \times 10^{-4}$	With Eq. (14b) in Ref. [1]	
		$V_{Trp} = -0.93 \times 10^{-4}$	Good in the back part of P_{Trp}	Agrees with Fig. 4D
		$V_{His} = -1.38 \times 10^{-4}$	Good in the back part of P_{His}	Agrees with Fig. 5D
5	40 mM pH 3.50 acetic buffer + 20 mM NaCl (+,α)||0.4 mM NaOH + 20 mM NaCl (−, β)	$V_{w\text{-}s} = 3.70 \times 10^{-4}$	With Eq. (17c) in Ref. [1]	
		$V_{Trp} = 1.15 \times 10^{-5}$	Poor in the back part of P_{Trp}	Agrees with Fig. 8A
		$V_{His} = 2.45 \times 10^{-4}$	Fair in the back part of P_{His}	Agrees with Fig. 6A
6	40 mM pH 3.50 acetic buffer + 20 mM NaCl (+,α)||2.0 mM NaOH + 20 mM NaCl (−, β)	$V_{w\text{-}s} = -5.25 \times 10^{-5}$	With Eq. (14b) in Ref. [1]	
		$V_{Trp} = -1.52 \times 10^{-4}$	Good in the back part of P_{Trp}	Agrees with Fig. 8B
		$V_{His} = -2.25 \times 10^{-4}$	Good in the back part of P_{His}	Agrees with Fig. 6B
7	40 mM pH 3.50 acetic buffer + 20 mM NaCl (+,α)||6.0 mM NaOH + 20 mM NaCl (−, β)	$V_{w\text{-}s} = -1.36 \times 10^{-4}$	With Eq. (14b) in Ref. [1]	
		$V_{Trp} = -1.15 \times 10^{-4}$	Poor at two sides of P_{Trp}	Agrees with Fig. 8C
		$V_{His} = -1.71 \times 10^{-4}$	Poor at two side of P_{His}	Agrees with Fig. 6C
8	40 mM pH 3.50 acetic buffer + 20 mM NaCl(+,α)||10 mM NaOH + 20 mM NaCl (−, β)	$V_{w\text{-}s} = -1.73 \times 10^{-4}$	With Eq. (14b) in Ref. [1]	
		$V_{Trp} = -0.93 \times 10^{-4}$	Poor at two sides of P_{Trp}	Agrees with Fig. 8D
		$V_{His} = -1.38 \times 10^{-4}$	Poor at two sides of P_{His}	Agrees with Fig. 6D

[a] The computation conditions are given in Fig. 4 in Ref. [1].

validity of theoretical results achieved in Ref. [1]. The experimental results achieved in this paper, coupled with the theoretical and experimental results in Refs. [1–4], hold evident significance for understanding of relevant stacking mechanism and has potential uses in the design of MRB-based stacking method of zwitterion samples, such as low abundance peptides and proteins, in CE.

2. Experimental

2.1. Chemicals

Acetic acid (AR), sodium acetic (AR), hydrochloric acid (AR), sodium chloride (AR), ammonia water (AR), dimethyl sulfoxide (DMSO, AR), L-tryptophan (Trp, Chrom pure) and L-histidine (His, Chrom pure) were purchased from the Shanghai Chemical Reagent Company (Shanghai, China). Sodium hydroxide (Guaranteed Reagent grade, GR) was from the Shanghai Zhungong Reagent Factory (Shanghai, China). The basic physical chemical parameters of His and Trp [5], which will be used for the calculation of mobility, are given in Table S1.

2.2. Apparatus

A high performance capillary electrophoresis (HPCE) (ACS 2000, Beijing Cailu Instrumental Co., Beijing, China) was used. The HPCE was equipped with a digital electric power supply (up to constant voltage 30 kV), a HW-2000 Chromatography Workstation and an UV–vis detector (double light beams, λ = 200–740 nm, set at 214 nm) which could perform scanning of wavelength from 200 to 740 nm. The fused-silica capillary (the Factory of Yongnian Optical Fiber, Hebei, China) with total length 50 cm, effective length 42 cm and I.D. 75 μm was employed. The runs were carried out under 22 °C. Before each run, the new capillaries were conditioned by rinsing with 1.0 M NaOH for 20 min, ultra-pure water for 10 min, 1.0 M HCl for 20 min and running buffer for 30 min, in order. An ultra-pure Water System (SG Wasseraufbereitung und Regenerierstation Gmbh, Germany) was used to produce the ultra pure water with conductivity down to 0.055 μS/m. If necessary, a conductance meter was used for specific conductivity of a buffer or sample solution.

2.3. Buffer and sample matrixes

The running buffers was 40 mM pH 3.50 acetic buffer with or without salt NaCl [6–8] (details see Table S2). Numerous different sample matrixes were prepared. The first kind is the normal sample matrix prepared with the 40 mM pH 3.50 acetic buffer (containing 5 μg/mL His, 1.0 μg/mL Trp and 0.03‰ DMSO). The second is the strong alkaline sample prepared with strong alkali NaOH shown in Table S3. The third is the weak alkaline sample with ammonia water (see Table S4). Before being used, the buffers and samples were degassed and centrifuged at 5,000 rpm 10 min if necessary. In order to investigate the zwitterionic stacking by MRB more clearly and effectively, some sodium chloride was added into sample buffers or solution to eliminate the stacking effect caused by the field-amplified sample injection (FASI). The treatment is effective as shown by these conductivity data in Table S1–S4.

2.4. General stacking procedure of transient MRB

Firstly, the anode end of the capillary was inserted into an alkaline salt sample, then the 15 mbar pressure was used for the injection of samples. Secondly, the sample vial was displaced with the anodic vial containing one of the running buffers in Table 1. After that, the power supply was turned on and a MRB was created between the running buffer (holding H^+) in the anodic vial and the alkaline sample (holding OH^-) at the cathodic side of the capillary as shown in Figs. S1 and S2 in Supplementary Material and Fig. 3 here. The created MRB is used for the stacking of zwitterion in CE, the mechanism of the stacking has been quantitatively discussed in Refs. [3,4].

During the experiments, the high-voltage power supply should be operated with extreme care to avoid the electric shock. In addition, one ought to avoid the harmful action of sodium hydroxide, ammonia water and hydrochloric acid to one's eyes and skin.

3. Results and discussion

3.1. CZE and effective mobilities of His and Trp

Fig. 1 shows the normal CZE electrophoregram of His and Trp, coupled with DMSO, *viz.*, the EOF marker, in sample plugs with dif-

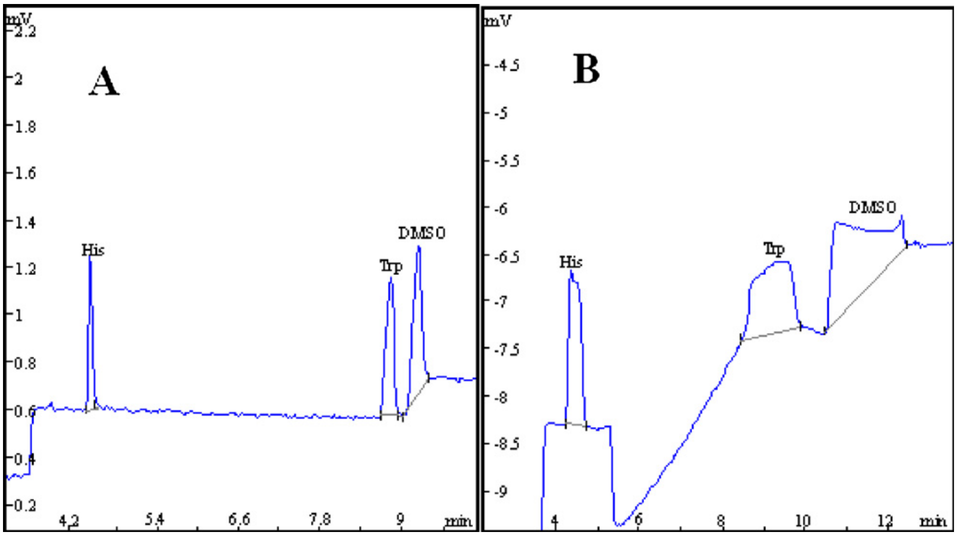

Fig. 1. The normal electrophoregrams of CZE of His, Trp and DMSO in the sample plugs with (A) 10 s and (B)120 s, 15 mbar injection times. Slight shoulder at the back side of His peak is observed. Conditions: normal sample: 5.0 μg/mL His, 1.0 μg/mL Trp and 0.03‰ DMSO dissolved in the running buffer; 50 cm total length (42 cm effective length) and 75 μm I.D. capillary; 15 kV and 7–9 μA electric current; pH 3.5 40 mM acetic running buffer; 22 °C.

ferent injection times of 15 mbar pressure. In Fig. 1A and B, the injection times are respectively 10 and 120 s. The comparisons between Fig. 1A and B evidently show that the peaks of His, Trp and DMSO become much wider, when the injection time of sample is increased to 120 s.

The mobilities of His and Trp under given conditions were detected from Fig. 1A. The effective mobilities of His and Trp in 40 mM pH 3.5 acetic buffer are respectively 2.71×10^{-8} and 0.13×10^{-8} $m^2 V^{-1} s^{-1}$. With the basic mobilities of His and Trp, as well as the physical chemical parameters in Table S1, one can calculate a series of their constituent mobilities at different pH values at low ionic strength (please see Table 2). These mobilities are the key parameters used in the comparisons between the boundary and zwitterion velocities given in Fig. S3–S5 in Supplementary Material and Table 1 herein.

3.2. Key conditions for stacking by MRB of weak acid and weak/strong alkali

Table 1, including the related experiments in Figs. 2, 4–6 and 8, displays the comparisons between the theoretical predictions and experimental results of the stacking of His and Trp by a transient MRB in CE. It is shown by No. 1 in Table 1 that in the system of 40 mM pH 3.50 acetic buffer (+, α)||0.4 mM NaOH + 20 mM NaCl (−, β), the velocity of Trp V_{Trp} in phase α is 1.05×10^{-4} m/s being obviously less than the boundary velocity $V_{w\text{–}s} = 1.18 \times 10^{-2}$ m/s. Hence it is predicted in Table 1 that the MRB cannot evidently stack the amino acid of Trp due to the rapid movement of MRB and the slow migration of Trp. The prediction was well proved by the experimental results in Fig. 2.

Fig. 2 shows that the 120 s plug of Trp sample is divided into two parts, the front and back half sample plugs (it is compared that the area of the two parts of Trp in Figs. 2A and 4A is equal to that of Trp peak in Fig. 4B–D, and if no Trp sample exists in the same alkaline solution, the two parts of Trp peak disappear). In contrast to the good stacking of front half sample plug, the back half is poorly stacked. This directly proves the poor stacking effect under the given conditions. The stacking of the front half sample plug is caused by the following mechanism. Fig. 3A shows that if an electric filed is applied, a transient MRB is created between OH^- in phase β and H^+ in phase α, the boundary moves towards the cathode with 0.0118 m/s, a very fast velocity. It is evaluated that the finish of MRB is around the middle point of sample plug, as shown in Fig. 3B. After the end of MRB, the back part of the sample plug is acidified, while the front part is weakly alkalized due to acetate ion in phase γ migrating into the part and chloride ion moving out from this section. Clearly a weak alkaline plug of sodium acetate is created within the front part of sample plug, the pH value of the plug is about pH 7–8 (the pH value of sodium acetate) being over the *p*I of Trp (=5.89) evidently, so the Trp molecules in the front part carry partial negative charge. At the same time, a new MRB is created between the hydrogen ion in the back part of sample plug and the sodium acetate in the front part of sample plug, as shown in Fig. 3B and C. The velocity of new boundary (≈1.5×10^{-4} m/s, if the concentration of sodium acetate in the front part is set at 10–15 mM) is much slower than that of the original boundary shown in Fig. 3A and near the velocity of Trp (=1.05×10^{-4} m/s), so the new boundary can lead to a strong stacking to the front half plug of Trp sample, as has been observed in Refs. [2–4]. This is a so-called derivative MRB-induced re-stacking. The re-stacking is directly proved by the good stacking of 120 s Trp sample plug in Fig. 2B in which 10 mM sodium acetate is used as sample buffer. Evidently, the facts that the original boundary causes poor stacking of the back part of Trp plug as shown in Figs. 2A and 4A and the new boundary leads to the good stacking of the front part of Trp plug observed in Figs. 2A and B and 4A, result in the cleavage of whole 120 s Trp sample plug.

No. 2 in Table 1 shows that if the concentration of NaOH is increased to 2.0 mM, the MRB velocity of 2.61×10^{-3} m/s is still high. So it is predicted that the poor stacking of Trp is still present in the back part of Trp peak. This prediction is well manifested in Fig. 4A in which the cleavage of sample plug is still present. When the concentration of NaOH is set at 6.0 mM, the MRB velocity decreases to 3.43×10^{-4} m/s being around the Trp velocity 1.05×10^{-4} m/s. Theoretically, a fair stacking of Trp plug occurs in the back part of the peak. The theoretical analysis is in agreement with the results of Fig. 4B (120 s injection) and 4C (60 s injection). The comparison between 4B and 4C shows that the front and back plugs of Trp, including His, can be acted as an excellent self-evidence to reveal the good MRB-based stacking of zwitterion if 120 s (Fig. 4B), but not 60 s (Fig. 4C) injection time is used. Hence, 120 s injection time is applied to the all experiments of MRB stacking (except for Figs. 4C and 10). At last, if 10 mM NaOH, including 20 mM NaCl, is used as sample solution as given in No. 4 in Table 1,

Table 2
The mobilities of His and Trp in different pH solutions at low ionic strength.

Zwitterion	Mobility ($\times 10^{-8}$ m^2 V^{-1} s^{-1})									
	Acetic buffer[a]	NaOH (mM)				Ammonia (mM)				Formate buffer[b]
		0.4	2.0	6.0	10	1.0	2.0	5.0	10	
His[c]	2.71	−2.62	−2.71	−2.71	−2.72	−2.43	−2.50	−2.58	−2.63	–
Trp[c]	0.13	−1.80	−1.89	−1.89	−1.89	−1.58	−1.66	−1.75	−1.79	0.92

[a] The running buffer of pH 3.50 40 mM acetic buffer used in Fig. 1.

[b] The running buffer of pH 2.85 30 mM formate buffer used in Fig. 12.

[c] The mobility of mono-valence positive amino acid, e.g., Trp, is assumed to be equal to the negative mobility of mono-valence negative amino acid approximately, namely there exist $m_{z,+} \approx -m_{z,-}$. The treatment has been proved by the results in Table 1 in Ref. [8] as early as in 1962 by Svensson.

the MRB velocity is -0.04×10^{-4} m/s towards the anode, at the same time the velocity of Trp in phase β is -0.93×10^{-4} m/s. The absolute velocity of Trp is much faster than that of MRB. Thus a good stacking in the back part of Trp peak is observed in Fig. 4D.

The amino acid of His was also used as a test example of stacking conditional investigations by the transient MRB. The comparisons of the velocities of the boundary and His are also performed in Nos. 1–4 in Table 1. It is obviously demonstrated that the predictions for His stacking by MRB are all in agreement with the experiments in Fig. 5A–D. The comparisons among the results of Fig. 5A–D show the gradual positive stacking effect to His sample. The results agree with the predictions of Nos. 1–4 for His stacking in Table 1. The stacking of His holds some difference as compared with that of Trp in the same system. It is clearly shown in Fig. 5 that some small peaks of His appear in front of the main peak of His. This is mainly induced by the presence of some positively charged His molecules near phase γ when the hydroxyl ion leaves the zone near phase γ rapidly as shown in Fig. S1 and S2.

The systems of Nos. 5–8 in Table 1 are respectively originated from that of Nos. 1–4 in Table 1 by adding 20 mM NaCl into 40 mM pH 3.50 acetic buffer. Hence, the MRB system of No. 5 is 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||0.4 mM NaOH + 20 mM NaCl + 5 μg/mL His (−, β). It is calculated that the velocity of His (=2.45×10^{-4} m/s) is slightly less than the MRB velocity (=3.70×10^{-4} m/s). Based upon the comparison, it is analyzed that a fair stacking of His can be observed in the back part of His peak. This analysis was well proved by the result in Fig. 6A. The prediction for His stacking in No. 6 of Table 1 is highly verified by the result in Fig. 6B.

According to the comparisons of Nos. 7 and 8 for stacking of His, the MRB-based stacking to the back part of His sample plug is poor. At the same time the stacking to the front part of His peak is poor due to the fast electro-migration of hydroxyl ion in phase β near boundary βγ. Hence one can conclude that the stackings of His peak at both parts are poor. This conclusion is evidently confirmed by the results in Fig. 6C and D. The two figures also show the slight stacking to the His plug in the middle part of the peak, rather than the left and right parts of His sample plug. The slight stacking of His is caused by the mechanism of transient FASI greatly contributed by Haglund and Tiselius [9] and Chien and Burgi [10–13], as will be shown in Fig. 7.

In the two electrolytic systems of Nos. 7 and 8, the boundary moves towards the anode shown in Fig. 7A. After the end of transient MRB, the 40 mM pH 3.50 acetic buffer was neutralized as

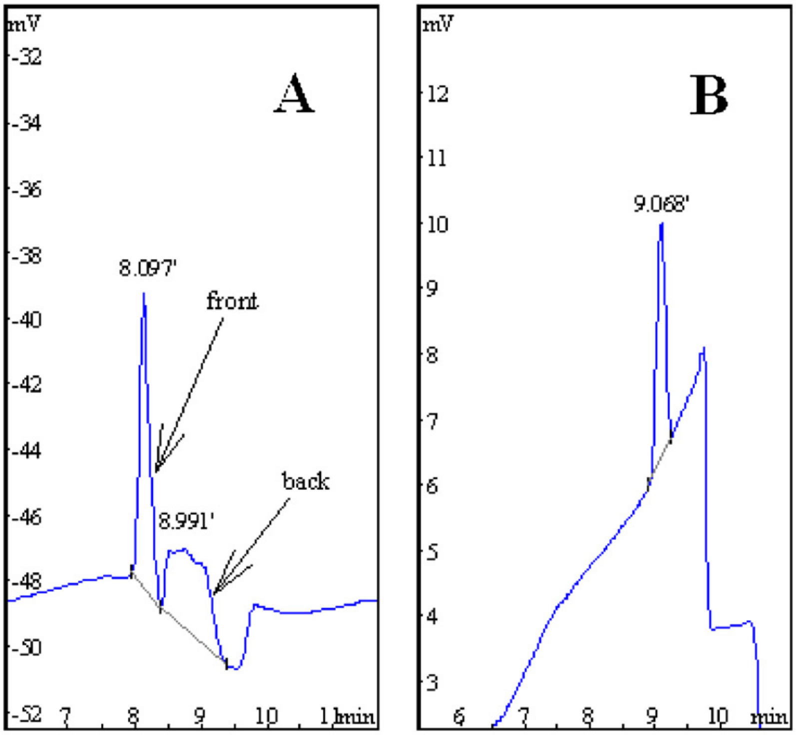

Fig. 2. The stacking of Trp sample plug in the systems of (A) 40 mM pH 3.50 acetic buffer (+, α)||0.4 mM NaOH + 20 mM NaCl + 5.0 μg/mL Trp (−, β) and (B) 40 mM pH 3.50 acetic buffer (+,α)||10 mM sodium acetate + 1.0 μg/mL Trp (−, β). The conditions are the same as those in Fig. 1.

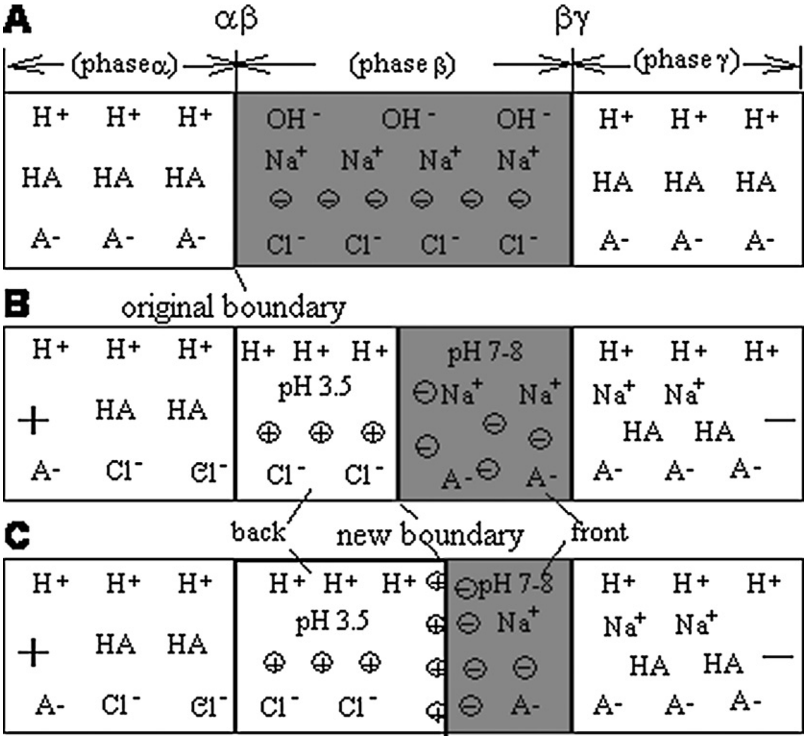

Fig. 3. The mechanisms of the first weak and second strong stacking, and the cleavage of Trp sample plug during and after a transient MRB formed with pH 3.50 40 mM acetic buffer and 0.4 mM NaOH. HA = acetic acid un-ionized, the hard symbols of + and − indicates the anode and cathode respectively, other symbols see the text.

∼40 mM sodium acetate. The zone of ∼40 mM sodium acetate, coupled with 20 mM NaCl in the same zone, form high conductivity, in contrast to the low conductivity zone of 20 mM NaCl zone derived from original phase β. The low conductivity zone of 20 mM NaCl leads to a transient FASI of His in the middle part of sample plug as shown in Fig. 7B. Owing for the rapid electro-migration of hydrogen ion in phase α, the 20 mM NaCl zone was acidified, and this leads to the rapid elimination of FASI. So the slight stacking occurs in the middle part of His sample plugs shown in Fig. 6C and D. This is a so-called transient FASI-induced re-stacking. This kind of transient FASI-induced re-stacking is also observed in Fig. 6B as indicated by the arrow. Clearly, the peak of FASI stacking is combined with the

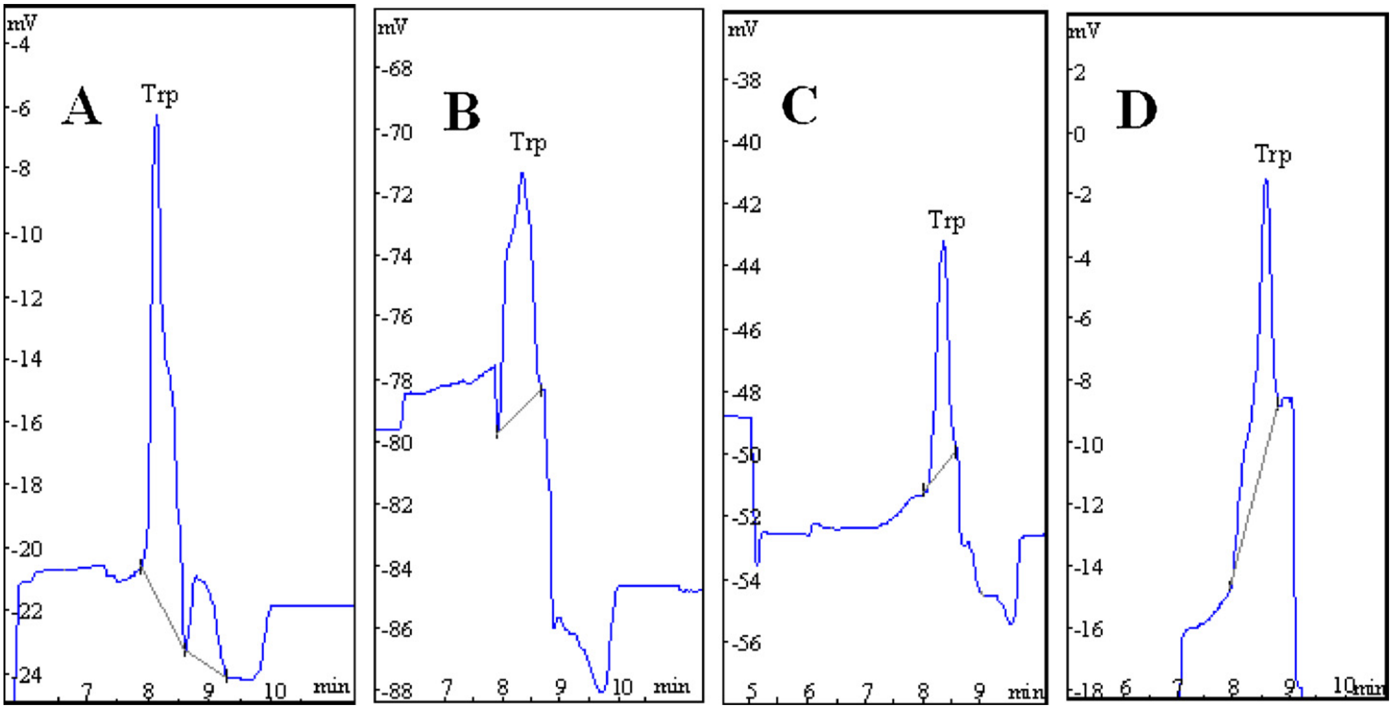

Fig. 4. The stacking of Trp in the system of (A) 40 mM pH 3.50 buffer (+,α)||2.0 mM NaOH + 20 mM NaCl + 5.0 μg/mL Trp (−, β) (120 s injection), (B) 40 mM pH 3.50 acetic buffer (+,α)||6.0 mM NaOH + 20 mM NaCl + 5.0 μg/mL Trp (−, β) (120 s injection), (C) being the same as that of B, but 60 s injection, and (D) 40 mM pH 3.50 acetic buffer (+, α)||10 mM NaOH + 20 mM NaCl + 5.0 μg/mL Trp (−, β) (120 s injection). The other conditions are the same as those in Fig. 1.

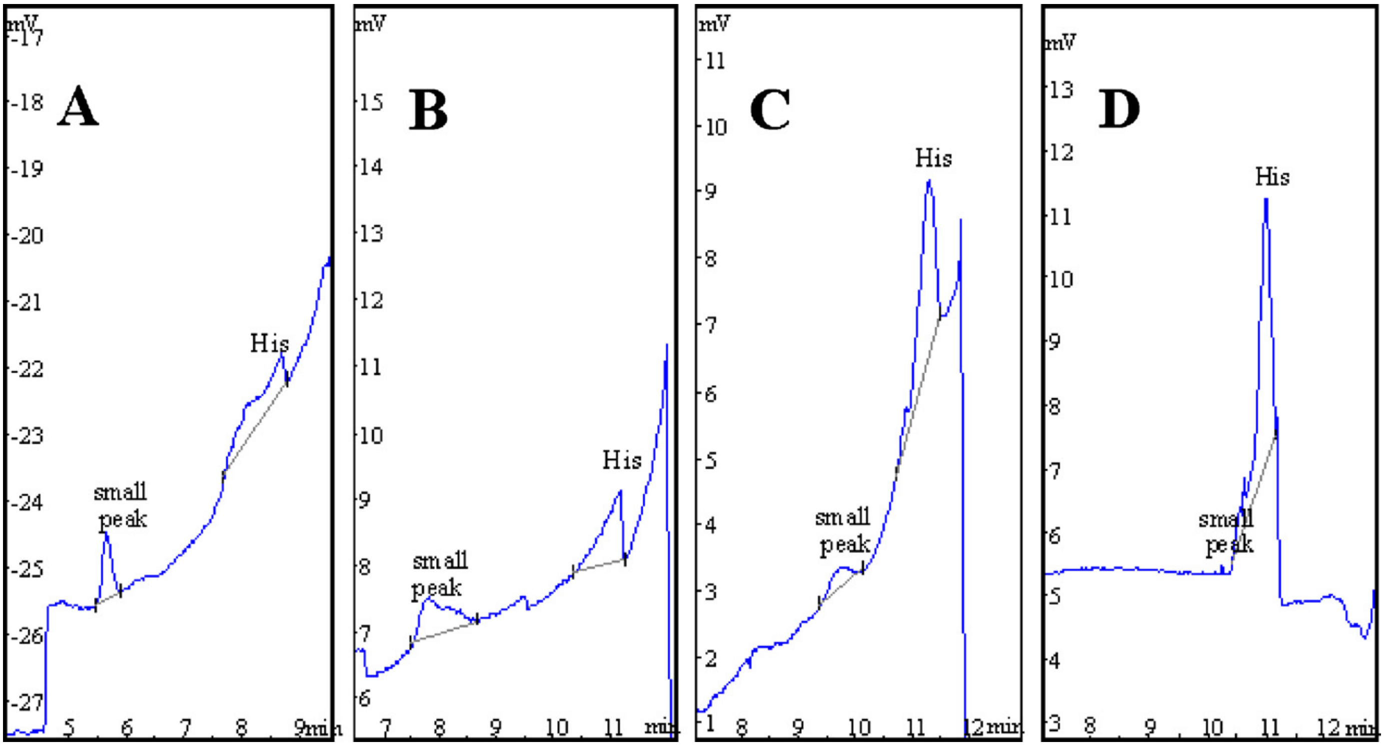

Fig. 5. The MRB stacking of 5.0 μg/mL His sample plug (120 s injection) in the system of (A) pH 3.50 40 mM acetic buffer (+, α)||0.4 mM NaOH + 20 mM NaCl (−, β); (B) pH 3.50 40 mM acetic buffer (+, α)||2.0 mM NaOH + 20 mM NaCl (−, β); (C) pH 3.50 40 mM acetic buffer (+, α)||6.0 mM NaOH + 20 mM NaCl (−, β); (D) pH 3.50 40 mM acetic buffer (+, α)||10 mM NaOH + 20 mM NaCl (−, β). The other conditions are the same as those in Fig. 1.

peak of strong MRB stacking to the back part of His plug. The kind of transient FASI-induced re-stacking is also found during the stacking of Trp with the same electrolytic systems as will be discussed below.

The comparisons of MRB and Trp velocities are shown in Nos. 5–8 of Table 1. The predictions are in accordance with these comparisons and the experimental results in Fig. 8. In contrast to the back part of His plug in Fig. 6C and D, the lengths of the back part

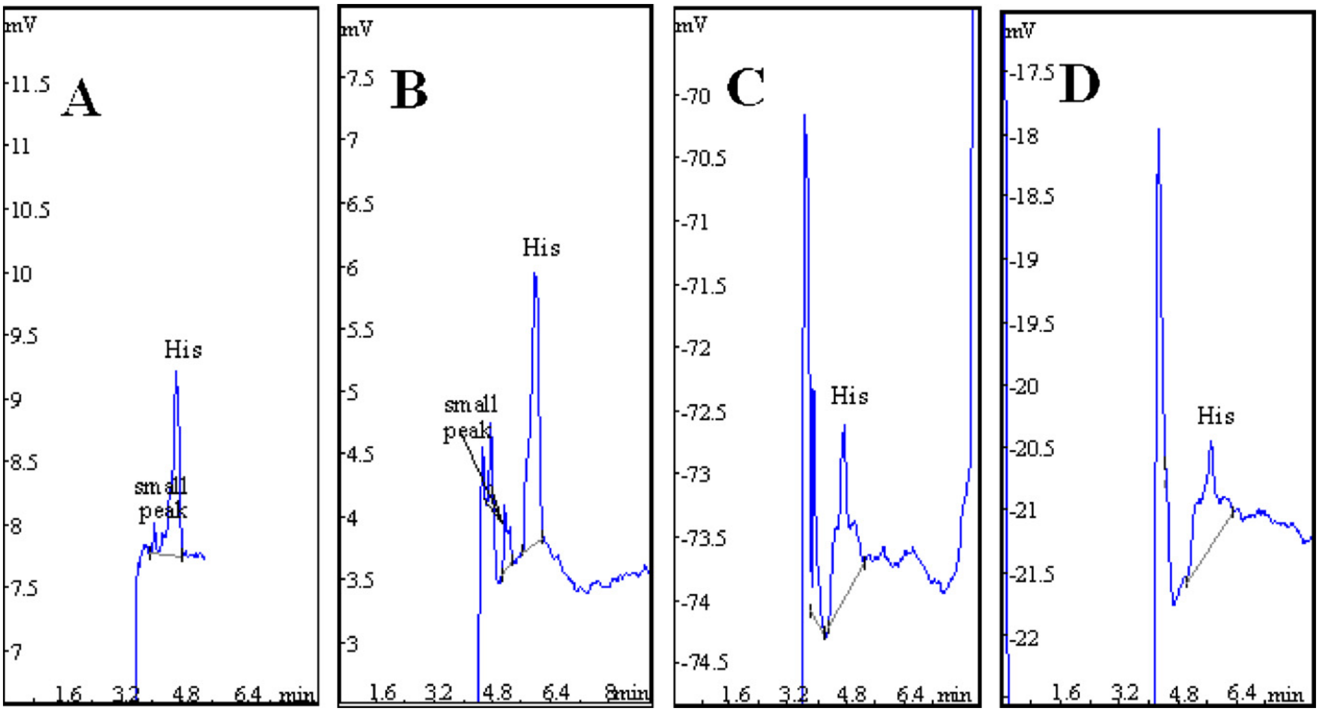

Fig. 6. The MRB stacking of 5.0 μg/mL His in the system of (A) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||0.4 mM NaOH + 20 mM NaCl (−, β); (B) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||2.0 mM NaOH + 20 mM NaCl (−, β); (C) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||6.0 mM NaOH + 20 mM NaCl (−, β); (D) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+,α)||10 mM NaOH + 20 mM NaCl (−, β). The arrow in (B) indicates FASI stacking in middle part of plug. The other conditions are the same as those of Fig. 1.

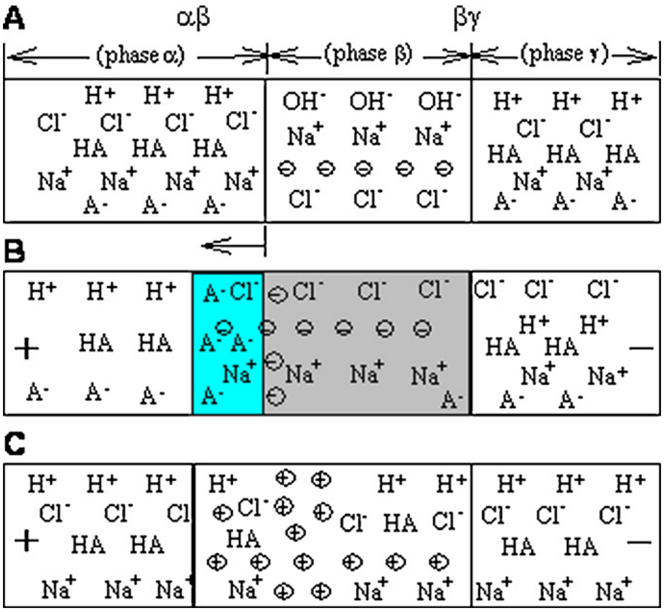

Fig. 7. The mechanism of the FASI stacking of His and Trp occurring in the middle part of whole sample plug in the system of 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||6.0 (or 10) mM NaOH + 20 mM NaCl (−, β) after the end of MRB stacking. The arrow indicates the movement of MRB.

of Trp plug in Fig. 8C and D are short, the heights are much low. The poor stacking of the back part of Trp is due to the slow migration of Trp towards the anode. As shown in No. 7 of Table 1, the Trp velocity in the alkaline solution is -1.15×10^{-4} m/s, which was slower than that of His in the same solution (=-1.71×10^{-4} m/s). Conversely, this poor stacking of the back part of Trp, together with FASI, causes great stacking in the front part of Trp and much high stacking in the middle part of the plug.

3.3. Front alkali plug is needed for the complete stacking if strong alkali is used to form MRB

Obviously, if the strong electrolyte (e.g., NaOH herein) is used for the stacking of the zwitterions of Trp and His, the transient MRB cannot completely stack the zwitterions. As analyzed in Sections 3.3 and 4.2 in Ref. [1], one can inject an alkaline plug without the sample in front of the alkaline sample plug. The length of the alkaline plug, which can completely stack the sample plug, can be calculated with Eq. (14) as shown in Ref. [1]. Table S5 shows that the front alkaline plug is about 300 s for complete stacking of 120 s His injection. The quantitative computation is proved by the results shown in Fig. 9.

In Fig. 9A–D, the peaks of 120 s His plug become sharper and sharper if the injection times of front alkaline plug (0.4 mM NaOH + 20 mM NaCl without zwitterion) are increased from 0, to 60, to 120 and to 180 s. Fig. 9E and F further reveal that if the injection was increased to 240 and 300 s, a quite stacking of His plug is achieved. The results quantitative verify the predictions on the stacking of His in 120 s sample matrix by the front alkaline plug, as given in Table S5.

It is also computed in Table S5 that the front alkaline plug is injected up to 555 s for the complete stacking of 120 s injection of Trp sample. It is difficult to perform 555 s injection of front alkaline plug for the complete stacking of 120 s Trp sample plug under the given conditions. Hence, the sample injection time of Trp is decreased to 60 s. According to the computation with Eq. (14) of Ref. [1], the injection time of front alkaline plug is about 278 s which can lead to complete stacking to the Trp plug of 60 s injection. The computation is demonstrated by the experiments in Fig. 10.

As shown in Fig. 10A–D, the peaks of 60 s Trp plug become thinner gradually if the injection times of front alkaline plug without zwitterion increase from 0, to 60, to 120 and to 180 s. It is obviously revealed in Fig. 10E and F that a good stacking of Trp sample plug is present if the injection time of front alkaline plug is set at 240 and 300 s. The experiments well manifest the computation on the stacking of Trp in 60 s sample plug as shown by Table S5.

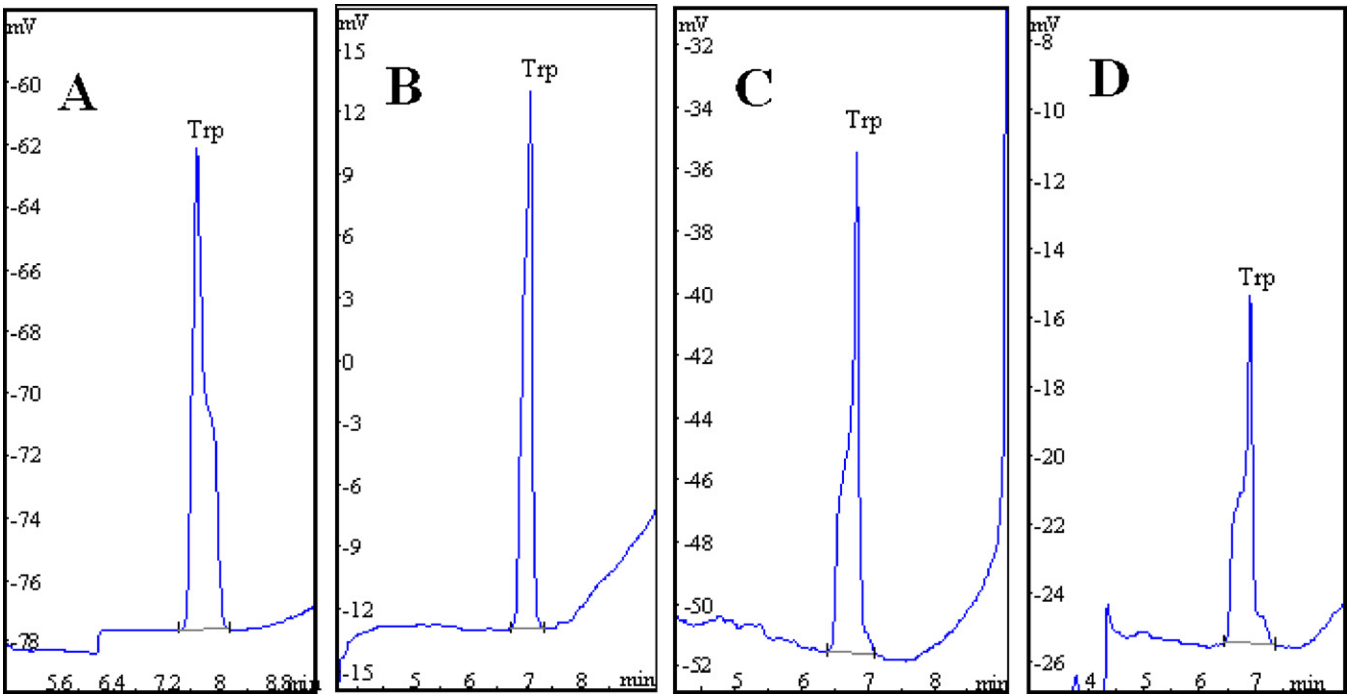

Fig. 8. The MRB stacking of 5.0 μg/mL Trp in the system of (A) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||0.4 mM NaOH + 20 mM NaCl (−, β); (B) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||2.0 mM NaOH + 20 mM NaCl (−, β); (C) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||6.0 mM NaOH + 20 mM NaCl (−, β); (D) 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||10 mM NaOH + 20 mM NaCl (−, β). The other conditions are the same as those of Fig. 1.

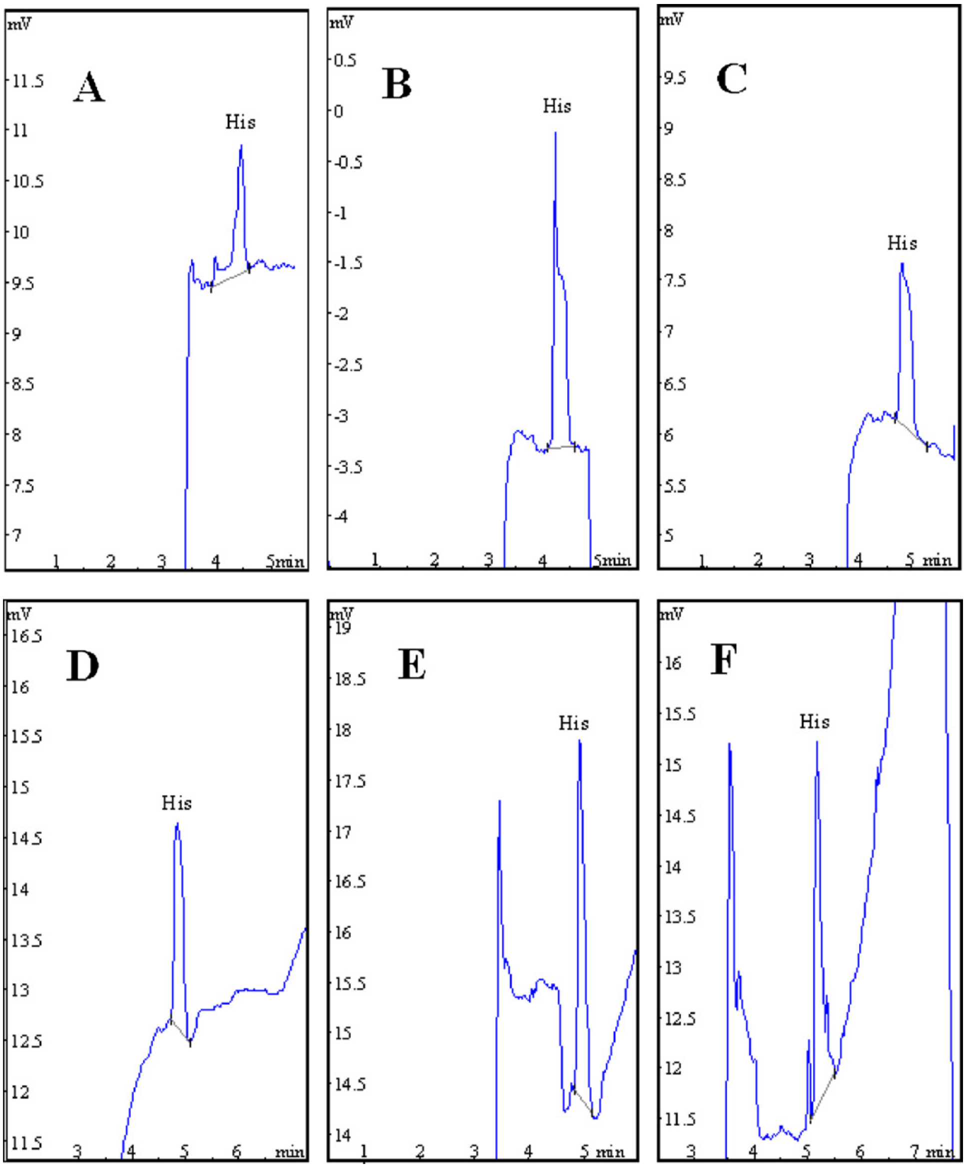

Fig. 9. The complete MRB stacking of 5.0 μg/mL His sample plug (120 s injection) by the front alkaline plug L_x = A: 0; B: 60; C: 120; D: 180; E: 240 and F: 300 s 15 mbar sample injection in the system of pH 3.50 40 mM acetic buffer + 20 mM NaCl (+, α)||0.4 mM NaOH + 20 mM NaCl (−, β). The other conditions are the same as those in Fig. 1.

Clearly, the predictions in Ref. [1] are highly proved by the experimental results of Figs. 9 and 10. Hence, it is concluded from the discussions above that a front alkaline plug without the zwitterion analyte ought to be injected for the complete stacking of zwitterion in the sample plug. The length of front alkaline plug can be predicted with the developed theory in the accompany paper [1].

3.4. *No front alkaline plug is needed for stacking if weak alkali is used to form MRB*

However, no front alkaline plug is needed for the complete stacking if weak alkali with enough high concentration is used for the preparation of sample matrix. Table S5 reveals that if 5.0 mM and 10 mM ammonia are used to prepare the sample of His, the lengths of front alkaline plugs ($L_{C.X}$) are respectively −27 s and −60 s. Unlike the positive values of $L_{C.X}$ for His and Trp in 0.4 mM NaOH in Table S5, the negative values of $L_{C.X}$ indicate that no front alkaline plug is needed for the stacking of His in ammonia. The theoretical result is verified by the experiments in Fig. 11 and Fig. S6.

Fig. 11A manifests the stacking of His by the MRB formed with the systems of 40 mM pH 3.50 acetic buffer + 10 mM NaCl (+, α)||1.0 mM ammonia + 20 mM NaCl (−, β). It is shown in Fig. 11A that the back part of His plug is not stacked evidently while the front part is highly stacked due to the similar re-stacking induced by derivative MRB as has been shown in Fig. 4A. The poor stacking to the back part of His plug is caused by the following reasons. It is calculated in Fig. S3A that if the ammonia is set at 1.0 mM, the velocity of MRB is much faster than that of His. Hence the MRB cannot stack the back part of His plug as observed in Fig. 11A. Fig. 11B indicates the stacking to the back part of His plug is better than that of Fig. 11A, but still incomplete stacking as indicated by the slight shoulder of His peak. This is also caused by the fact the velocity of MRB is slightly faster than that of His as shown in Fig. S3A.

However, a good stacking occurs if 5.0 mM or 10 mM ammonia is used to the MRB, as be clearly observed in Fig. 11C and D and Fig. S6. The good stacking can be illuminated by the computation in Fig. S3A. As shown in Fig. S3A, the velocity of MRB is less than that of His if 5.0 mM or 10 mM ammonia is used to prepare the sample matrix of His. The mechanism of good stacking is sim-

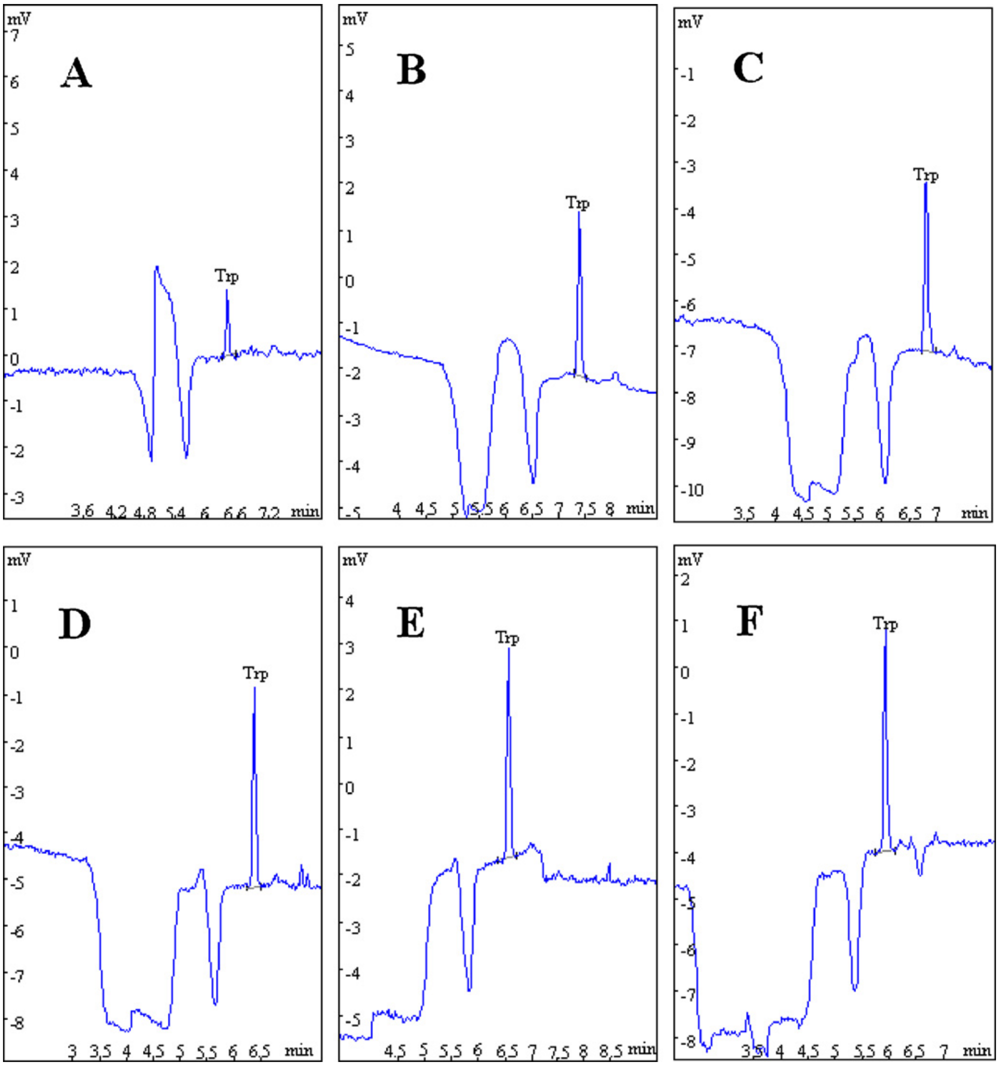

Fig. 10. The complete MRB stacking of 5.0 μg/mL Trp sample plug (60 s injection) by the front alkaline plug L_x = A: 0; B: 60; C: 120; D: 180; E: 240 and F: 300 s 15 mbar sample injection in the system of pH 3.50 40 mM acetic buffer + 20 mM NaCl (+, α)||0.4 mM NaOH + 20 mM NaCl (−, β). The other conditions are the same as those in Fig. 1.

ilar to that in previous work [3,4]. It is clearly revealed in Fig. 11C and D that no front alkaline plug is needed for the stacking of His if 5.0 mM or 10 mM ammonia is used for the preparation of His sample.

The computation in Table S5 further shows that the values of front alkaline plug are negative ones if 60 mM or 100 mM sodium formate is used to create the MRB system. Manifestly, no front alkaline plug is required for the complete stacking of Trp under the given conditions. The theoretical conclusion has been well conformed in the previous papers [2–4].

Hence, the computations in Table S5, and the experimental results in Fig. 11 and Refs. [2–4] obviously demonstrate that front alkaline plug is not needed for the stacking of His if alkali with enough high concentration is used for the preparation of sample matrix.

3.5. Dramatic influence of salt on stacking by MRB of weak acid and strong alkali

Not only is no front alkaline plug without analyte needed for the complete stacking by a MRB (see Section 3.4), but also the MRB system can strongly antagonize the influence of high salt existing in the boundary system if weak acidic and alkaline buffer are used to form the boundary system. It was firstly observed in the stacking of Trp by the MRB system of formic buffer (+,α)||sodium formate + 0–120 mM NaCl + Trp (−,β) that up to 120 mM sodium chloride has no evident negative influence on the MRB-based stacking of Trp [2]. It was further revealed in Refs. [3,4] that the weak influence of high salt in the MRB system was originated from the fact that the existence of salt has much weak effect on the fluxes of hydrogen ion in the formic buffer and hydroxyl ion in the sodium formate.

Fig. S3 also indicates that (1) if less than 3.5 mM ammonia in the sample is used to create the boundary, the MRB cannot effectively stack His in the sample matrix, and (2) the high concentration salt of sodium chloride has much weak effect on the stacking of His sample plug by the MRB formed with formic buffer and ammonia. Evidently, the experiments in Fig. 11 well demonstrate the first theoretical conclusion, and the results in Fig. 12 clearly manifest the second one. Thus, there is weak influence of salt on MRB-based stacking if a boundary is created with weak acid and weak alkali.

However, dramatic influence of salt on the MRB-induced stacking is present if the boundary is formed with weak acid and strong base, such as NaOH, used herein. Fig. S4 shows the comparisons among the velocities of His, Trp and MRB. In Panel A, the boundary system is 40 mM pH 3.50 acetic buffer (+,α)||0.40–12 mM NaOH + 20 mM NaCl + 5 μg/mL Trp (−, β) or 5 μg/mL His. The predictions of Panel A given in Nos. 1–4 in Table 1 herein are all

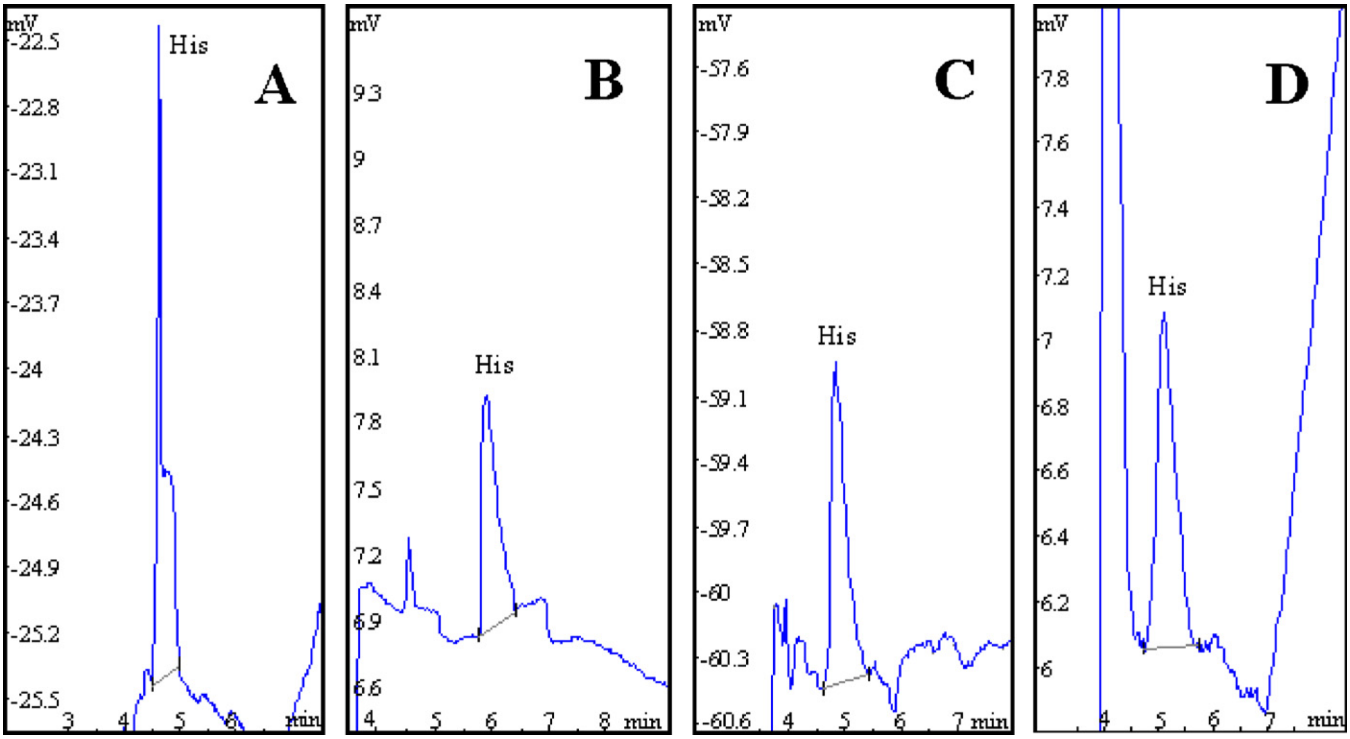

Fig. 11. The MRB stacking of +5.0 μg/mL His sample plug (120 s injection) in the system of pH 3.50 40 mM acetic buffer + 10 mM NaCl (+,α)||20 mM NaCl + A: 1.0 mM, B: 2.0 mM, C: 5.0 mM, D: 10 mM ammonia (−, β). The other conditions are the same as those of Fig. 1.

demonstrated by the experiments of Figs. 2A, 4A–D and 5A–D, respectively, as has been discussed in Section 3.2. In Panel B, the system is 40 mM pH 3.50 acetic buffer + 20 mM NaCl (+, α)||0.40–12 mM NaOH + 20 mM NaCl + 5 μg/mL Trp (−, β) or 5 μg/mL His. The theoretical computations and predictions shown in Nos. 5–8 in Table 1 herein are well verified by the experimental results of Figs. 6A–D and 8A–D, respectively. The only difference of Panel A and B is that the 20 mM NaCl is added into the 40 mM pH 3.50 acetic buffer in Panel B, rather than Panel A. Whereas, the 20 mM salt can induce the dramatic differences of MRB, His and Trp velocities, as clearly shown in Fig. S4A and B, and further results in great difference of MRB-based stacking efficiency as shown by Nos. 1–4 and Nos. 5–8 in Table 1 as well as the relevant experiments of Figs. 2–8 herein.

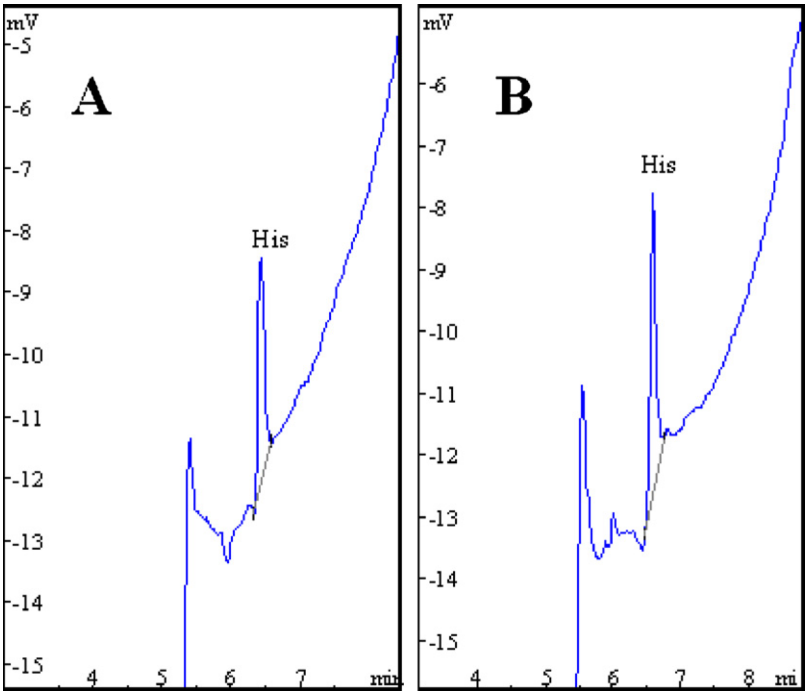

Fig. 12. The MRB stacking of +5.0 μg/mL His sample plug (120 s injection) in the systems of 40 mM pH 3.50 acetic buffer + 10 mM NaCl (+, α)||10 mM ammonia + (A) 0 mM NaCl, or (B) 80 mM NaCl (−, β). The other conditions are the same as those of Fig. 1.

4. Conclusion

From the experiments, comparative studies and discussions, we can achieve the following conclusions. At first, the comparisons between MRB and zwitterionic velocities hold key significances to the design of the MRB stacking of zwitterion, and for a good MRB-based stacking of zwitterion solute, the design on MRB velocity ought to be performed in accordance with the inequality of $0 < V_{\mathrm{weak}}^{\alpha\beta}(\mathrm{or}\ \left|V_{\mathrm{w-s}}^{\alpha\beta}\right|) \leq \bar{V}_{z+}^{\alpha}(\mathrm{or}\ \left|\bar{V}_{z-}^{\beta}\right|)$. The comparative studies of Nos. 4 and 6–8 in Table 1 show that the good stacking of zwitterion is achieved under the condition of boundary velocity towards the anode becoming much less than that of zwitterion (moving towards the anode), and the poor MRB stacking of zwitterion occurs under the condition of boundary velocity towards the anode being faster than or near the velocity of the negative zwitterion in phase β. Those comparisons of Nos. 1–3 and 5 in Table 1 verify that if the velocity of boundary in the direction of the cathode is much faster than that of zwitterion in phase α, MRB cannot well focus the zwitterion; conversely MRB can highly stack zwitterion. The experiments of Fig. 11 also illustrate the conclusion. Clearly, the results of Nos. 1–4 in Table 1 highly prove the predictions in Fig. S4A, those of Nos. 5–8 well demonstrate the theoretical calculations in Fig. S4B, and the comparisons of experiments (except for the much high concentration alkali, such as 20 mM ammonia and 120 mM sodium formate) in Fig. 11 accurately confirm the computations of Fig. S5. These results evidently unveil the importance of the design of MRB velocity.

Second, an enough front alkaline plug, $L_{\mathrm{C.X}}$, is necessary for the complete stacking of zwitterion, if the sample is prepared with a strong alkali like NaOH; conversely no front alkaline plug is needed if the sample is prepared with a weak alkali with enough high concentration and proper pH value. The comparisons between Figs. 9 and 10 well prove the theoretical predictions of Table S5. The experiments of Fig. 11, as well as those in Refs. [2–4], show no front alkaline plug is necessary if a weak alkali (such as ammonia and sodium formate) with enough high concentration and proper pH value is used to prepare the relevant sample matrix. The results herein and in Refs. [2–4] directly confirm the predictions of Table S5.

Third, the presence of salt in MRB has great influence on the stacking of zwitterion if strong alkali of NaOH is used to prepare sample matrix, but weak effect on the same stacking if weak alkali (e.g., ammonia and sodium formate) is used. The comparisons between Nos. 1–4 and Nos. 5–8 in Table 1 clearly shows dramatic effect of salt NaCl on the MRB stacking of zwitterion if a strong electrolyte, e.g., NaOH, is used to prepare the sample, and directly prove the dramatic prediction of Fig. S4. Conversely, the comparative investigations of Fig. S3 and Fig. 11 markedly demonstrate the much weak effect of salt on the MRB stacking of zwitterion if weak alkali is applied.

Hence, the theoretical predictions in Figs. S3–S5 are quantitatively confirmed by the experiments in this paper. The comparative studies mentioned above manifestly illustrate that a weak alkali holds incomparable superiority to a strong alkali when be used as the sample matrix.

Lastly, the experiments of this paper quantitatively prove the validity of the theory developed from MRB in Ref. [1]. Thus, the theory developed in Ref. [1] can accurately predict the experimental conditions and efficiency of stacking zwitterion of MRB-based stacking method.

Additionally, the so-called derivative MRB-based and transient FASI-induced re-stackings were also observed during the runs of MRB-based stacking with strong alkali used as sample solution, and the relevant mechanisms were briefly demonstrated with some theoretical computations and experiments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.01.062.

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