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Velocity Gap Theory Developed for Magnifying Resolutions without Changing Separation Mechanisms or Separation Lengths

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Separation techniques, such as chromatography and electrophoresis, form the basis in many fields and are continually developed for better separation efficiency. The efforts normally involve a new mechanism together with sufficient separation length. We develop a velocity gap theory to make things simple. The theory is based on the discovery that the velocity gap (VG) effect could enlarge the distance between two moving objects. Mathematical deduction certified that the resolution may be magnified infinitely without changing the separation mechanism or the separation length. DNA separation confirmed its practical feasibility by achieving 2−5 times higher resolution on a microchip. Our results indicate that VG effect could enlarge the distance between two moving objects and may potentially be utilized to ameliorate separation efficiency.

Chromatography and electrophoresis play a vital role as separation techniques in many fields, such as organic synthesis, pharmaceutical sciences, clinical chemistry and toxicology, and forensic science, etc.[1−4] They especially form the bone of modern biology.[5−7] These two kinds of techniques share the same feature that separation is the result of the speed-difference between two components. Separation scientists thus design a reverse process, which refers to the positive and the negative outcomes that are derived from executing a certain act, to achieve this speed-difference.[8−10] Bao et al. described an electrophoretically mediated microanalysis (EMMA) which involved two different forms of the same compound to allow enzyme assays in capillary electrophoresis.[11] Lambertus et al. demonstrated an enhanced separation with a combination of two columns which have different stationary phases. A pneumatic valve was used to avoid coeluting the components separated by the first column.[12] The separation effect is determined upon the difference degree by which the components are involved in the reverse process, i.e., the separation mechanism. A better separation effect normally requires adopting a new separation mechanism together with sufficient separation length. The work thus becomes professional and tedious. In addition, the sufficient separation length forms an obstacle to miniaturize the scale of separation techniques.[13−15] An example is given that a poorer separation capacity suffers when transferring an analysis from capillary electrophoresis to microchip electrophoresis (MCE) due to the relatively short separation channel.[16] Although some methods applied in MCE were effective, they were too special to be generalized. To address this problem, here we present a velocity gap (VG) theory which aims to magnify the separation resolution without changing separation mechanisms or separation lengths.

VELOCITY GAP THEORY

Concept of the VG Effect. The concept of VG is from the velocity change as components move in the background solution

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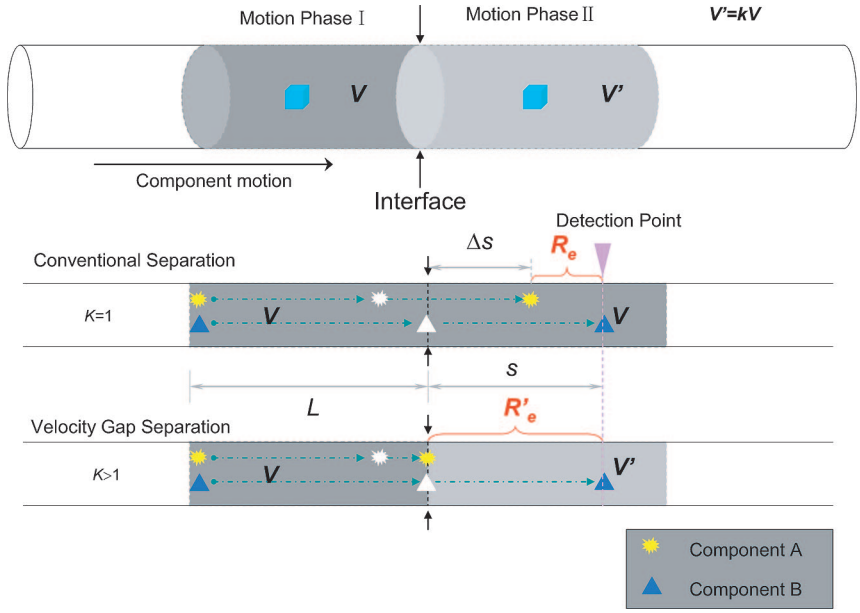

Figure 1. Schematic diagram of the velocity gap theory. An interface divides the background solution into motion phases I and II. The component drifts at the speed V in motion phase I and at the speed V' in motion phase II ($V' = kV$, $k > 1$). Assume two components are separated ($V_A < V_B$). When component B (explosion shape) arrives at the detection point, the distance between components A and B (triangle shape) represents the separation resolution. In conventional separation, the components drift at the same speed in both two motion phases ($k = 1$). Component B will drift at the speed V_B in motion phase II, and the resolution is R_e. In contrast, component B will drift at the speed V_B' ($V_B' = kV_B$, $k > 1$) in motion phase II in the VG separation. The resolution thus becomes R_e'. ΔS represents the enlarged resolution achieved by VG separation compared with a conventional separation. The blank explosion and triangle shapes indicate the positions of components in the midway.

(BGS). Instead of a relatively uniform motion in conventional separation techniques, an interface divides the BGS of VG separation into motion phase I and motion phase II (Figure 1). One component may migrate at different speeds in two motion phases: V in motion phase I and V' in motion phase II. The relationship between V and V' is expressed by eq 1.

$$V' = kV \tag{1}$$

where k is a coefficient. According to the range of the k value, three cases exist. When $0 < k < 1$, the component moves slower in motion phase II than in motion phase I. The dynamic pH junction technique belongs to this case, which utilizes a sharp reduction in an analyte's migration velocity for online preconcentration.[17] Sample concentration could also be achieved in this case.[18] When $k = 1$, the component moves at the same speed in both motion phases. The conventional separation techniques belong to this case. When $k > 1$, a component moves faster in motion phase II than in motion phase I. The VG effect is based on this case, and the separation efficiency could be improved with increasing the k value.

Theoretically Certifying VG Theory. Because the separation resolution is proportional to the distance between the two components, for ease of explanation, we use the distance between two components to represent the separation resolution. The VG effect in magnifying resolutions is analogous to the enlarged distance between two teams when handing over a baton in a relay race. As shown in Figure 1, two components, components A and B, are supposed to be separated. According to a separation mechanism, component A moves at the speed V_A and component B moves at the speed V_B ($V_A < V_B$). Assume that the two components start migrating at the same position and the distance from starting point to the interface is L, we could calculate the time taken for two components to arrive at the interface. Component A needs time t_A,

$$t_A = \frac{L}{V_A} \tag{2}$$

and component B needs time t_B,

$$t_B = \frac{L}{V_B} \tag{3}$$

Because component B moves faster than component A, when component A arrives at the interface, component B has already entered into motion phase II and drifted for time Δt,

$$\Delta t = t_A - t_B = \frac{V_B - V_A}{V_A V_B} L \tag{4}$$

assuming that component B happens to arrive at the detection point. The distance S is used to represent the resolution achieved by VG separation. As shown in Figure 1, since the components will accelerate their speeds at the interface ($k > 1$, $V' = kV > V$), component B will move at the speed V_B' ($V_B' = kV_B$) instead of V_B in motion phase II. The resolution (R_e') of VG separation is expressed by

$$R_e' = S = V_B' \Delta t = k \frac{V_B - V_A}{V_A} L \tag{5}$$

For comparison, the resolution (R_e) in conventional separation between the two components could then be expressed by

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$$R_e = S - \Delta S \quad (6)$$

where ΔS represents the distance that component A migrated in motion phase II when component B arrived at the detection point. Comparing eqs 5 and 6, we could obtain the relationship between R_e' and R_e as

$$\Delta S = R_e' - R_e = (k - 1)\left(1 - \frac{V_A}{V_B}\right)L \quad (7)$$

where ΔS is for the enlarged resolution achieved by VG separation compared with conventional separation. L is a constant. When the separation mechanism is determined, $(1 - V_A/V_B)$ also becomes a constant of m ($0 < m < 1$ because $V_A < V_B$). The only changed factor is $(k - 1)$. $V' > V$, so $k - 1 > 0$. With an increase in the value of k, the resolution achieved by VG separation will thus be magnified.

Characters of the VG Effect in Separation. In conclusion, several points should be emphasized in the VG separation: (1) Since k is a parameter decided by the interface of two motion phases and could theoretically increase up to infinity, the separation resolution could potentially be magnified infinitely if an appropriate interface exists. (2) The VG effect is based on enlarging the distance between two moving objects. It thus has a high compatibility with most separation techniques, such as chromatography and electrophoresis. (3) The VG effect occurs at an interface which is determined only by the characters of motion phases, so the separation length is not a prerequisite condition. (4) When compared with conventional separations, less running time is required in the VG separation because the components are accelerated when they enter into motion phase II. (5) The VG theory is simple but effective.

EXPERIMENTAL SECTION

Manipulating the VG Separation in MCE. DNA fragments were labeled with TO-PRC-3 before analysis for detection. MCE was performed on poly(methyl methacrylate) chips with a 30 mm efficient separation length in a HITACHI SV1210. Microchip electrophoresis specifications were as previously described.[19] Briefly, the poly(methyl methacrylate) chips consisted of a simple cross channel 100 μm wide and 30 μm deep. Distances from the channel intersection to the sample (S), sample waste (SW), buffer (B), and buffer waste (BW) reservoirs were 5.25, 5.25, 5.75, and 37.5 mm, respectively. The effective separation channel length was 30 mm. DNA ladder (100bp) was chosen as the sample (Takara/Shiga, Japan). The running buffer was Tris-Borate-EDTA (TBE) (Sigma, St. Louis, MO). Polymer methyl cellulose (MC) (Sigma, St. Louis, MO) was solved in the running buffer as the gel matrix. The running buffer was loaded into the microchannel by applying pressure via a syringe (Figure 2). In step A, 0.5%MC, ×1TBE buffer was injected into the channel to cover the area of the channel except for the efficient length. The interface between this buffer and motion phase II led to sample concentration before entering the separation channel. In step B, 0.5%MC, ×4TBE buffer was injected as motion phase I. In step C, 0.5%MC, the lower concentration buffer, i.e., ×2, ×1, ×0.5, ×0.25, and ×0.1TBE, was injected as motion phase II. In step D, reservoir B and SW were filled with 0.5%MC, ×1TBE buffer and reservoir S was filled with sample solution. The sample loading was carried out by applying 300 V voltages in the SW reservoir, and other reservoirs were kept grounded. During the separation process, a 750 V voltage was applied in the BW reservoir and kept the B reservoir grounded. Appropriate voltage was added in the S and SW reservoirs to avoid sample contamination. All data of the second run was adopted.

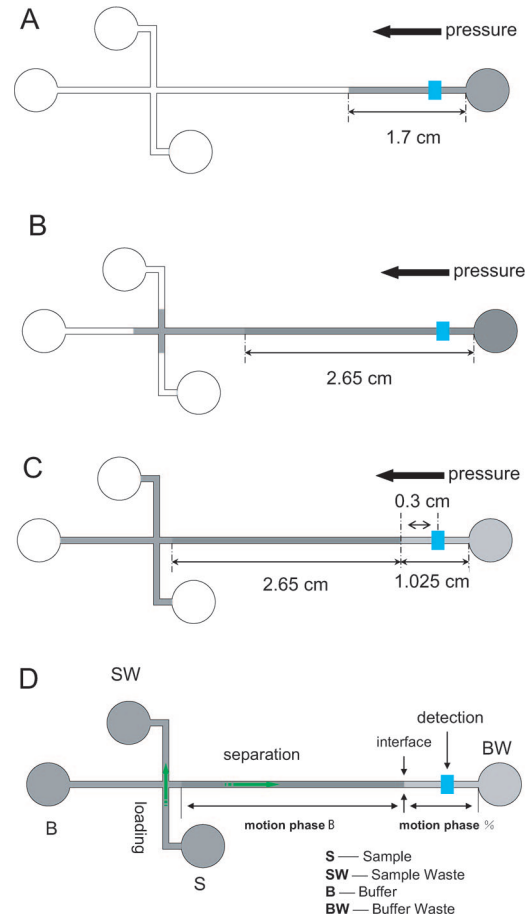

Figure 2. Schematic diagram of the VG separation in MCE: (A) First, 0.5%MC, ×1TBE buffer was injected into the microchannel. (B) Second, 0.5%MC, ×4TBE buffer was injected into the microchannel as motion phase I. (C) Third, 0.5%MC, the lower concentration TBE buffer was injected into the microchannel as motion phase II. (D) MCE analysis.

RESULTS AND DISCUSSION

Practical Feasibility of VG Theory in DNA Separation. In order to certify the VG theory in practical application, we separated a mixture of DNA fragments in microchip electrophoresis (MCE) as an example. The mixture of DNA fragments consists of 11 double-stranded DNA fragments with sizes of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1500 base pairs. Polymer methyl cellulose (MC) was added in TBE buffer to form a gel matrix. Separation was based on the sieving mechanism and performed on a 3 cm efficient length channel.

Designing Two Motion Phases with Different Concentrations of TBE Buffers. The design of two motion phases is based on a basic physical phenomenon that the electric current value is equal at any intersection in a series circuit. Two motion phases

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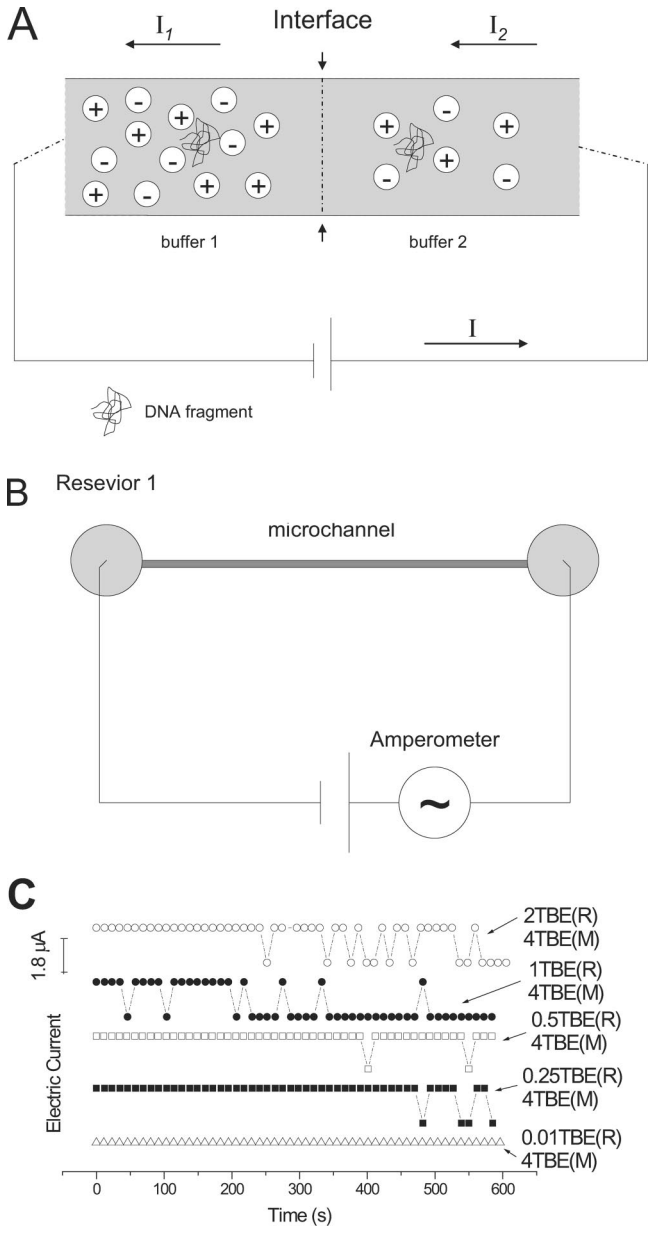

Figure 3. A design for creating two motion phases. (A) The two TBE buffer solutions, buffer 1 (C_1) and buffer 2 (C_2), are successively loaded into the microchannel ($C_1 > C_2$). The two ends are then connected with a direct electric power supply to form a series circuit. From eqs 8 and 12, negatively charged DNA fragments will drift at speed V_1 in buffer 1 and V_2 in buffer 2 ($V_1 < V_2$). Two motion phases are thus created by the two buffer solutions. (B) A series circuit was designed to confirming the existence of an interface. While ×4TBE buffer is filled in the microchannel, the lower concentration TBE buffer is filled in the two reservoirs, i.e., ×2TBE; ×1TBE; ×0.5TBE; ×0.25TBE; ×0.1TBE buffer, respectively. All buffer solutions contain 0.5%MC. (C) An amperometer is connected to monitor the change of electric current. Relatively stable electric currents in each case indicate that ion diffusion is limited and an interface between the two buffer solutions exists (R, reservoir buffer; M, microchannel buffer).

are substituted with two buffer solutions which are composed of TBE buffer and 0.5%MC polymer. The only difference of the two buffer solutions is their concentrations, i.e., buffer 1 is C_1 and buffer 2 is C_2 ($C_1 > C_2$). As shown in Figure 3A, two buffer solutions are consecutively injected into the microchannel to form a series circuit. When electric voltage is applied at the reservoirs, an electric current would be created in the microchannel ($I_1 = I_2 = I$). The electric current could be calculated from the following equation:

$$I = CAVQ \tag{8}$$

I is the electric current, C the number of charged particles per unit volume (or ion concentration), A the cross-sectional area of the microchannel, V the drift velocity, Q the charge of each particle. $I = I_1 = I_2$ and A and Q are constant values. So V is the reciprocal proportional to C. From eq 8, the drift velocity of charged particles (V) is expressed by

$$V = \frac{I}{AQ}\frac{1}{C} \tag{9}$$

The speed in buffer 1 is V_1:

$$V_1 = \frac{I_1}{AQ}\frac{1}{C_1} = \frac{I}{AQ}\frac{1}{C_1} \tag{10}$$

and the speed in buffer 2 is V_2:

$$V_2 = \frac{I_2}{AQ}\frac{1}{C_2} = \frac{I}{AQ}\frac{1}{C_2} \tag{11}$$

From eqs 10 and 11, we obtain the relationship of V_1 and V_2:

$$V_2 = \frac{C_1}{C_2}V_1 \tag{12}$$

$C_1 > C_2$, so $V_2 > V_1$. Thus two motion phases are achieved by loading two buffer solutions with different concentrations in the microchannel. Negatively charged DNA fragments drift faster in the lower concentration buffer solution (C_2) and drift slower in the higher concentration (C_1).

However, diffusion normally occurs at the interface of two solutions with different concentrations, i.e., ions in the solution of higher concentration have tendency to diffuse into the solution of the lower concentration until equilibrium. It is reasonable to doubt if the interface really exists.

Confirming the Existence of an Interface Between Two Motion Phases. Interestingly, we found that polymer MC in TBE buffer could limit the diffusion of ions caused by a concentration gradient. A series circuit was designed to confirm this conclusion (Figure 3B). The microchannel was filled with ×4TBE buffer, and two reservoirs were filled with diluted TBE buffer. TBE buffer contained 0.5%MC polymer. An amperometer was connected into the series circuit to monitor the electric current in the microchannel. If diffusion caused by concentration gradients happened, the ion concentration in the microchannel would decrease and so did the electric current. We thus could judge whether diffusion occurred or not by monitoring the conductance change. As shown in Figure 3C, while the buffer in the reservoirs varied from ×0.1TBE to ×2TBE, the electric current was kept relatively stable in each case. It indicated that polymer MC in TBE buffer could limit diffusion caused by ion concentration gradients. The reason remained unclear, but the high viscosity of polymer MC may be responsible for this phenomenon.

VG Effect in Separating DNA Fragments. The MCE was performed according to the description.[19] Controlled experiment filled in both two motion phases with 0.5%MC, ×4TBE buffer (*k*

= 1). Separation was carried out in conventional style in which the separation effect was only determined by the sieving capacity of the matrix. VG separation filled in motion phase I with ×4TBE buffer which was the same as conventional separation and filled motion phase II with diluted TBE buffer (Figure 2). The addition of 0.5%MC in buffer solutions was for two purposes: one was to limit ion diffusion which may occur at the interface of two motion phases. The other was to keep the same sieving capacity of BGS as a conventional separation. Thus, compared with conventional separation, VG separation did not change the separation mechanism. The only changed parameter was the velocities of DNA fragments when they enter in motion phase II, i.e., VG. If k was used to represent C_1/C_2 in eq 12, the value of k was proportional to VG. The resolutions between each pair of DNA fragments were gradually magnified when VG was enlarged with increasing k from 2 to 40 (parts A and C of Figure 4). Dilute TBE buffer showed no contribution to the enhanced resolutions (Figure 4B). The results indicated that the VG effect caused the separation resolutions to be magnified. We did not continue to increase the k value because diffusion phenomenon became strong when the buffer concentration in motion phase II was lower than 0.1×TBE (data not shown). The resolutions achieved by VG separation in MCE ($k = 40$) were comparable to those obtained by capillary electrophoresis (CE).[20]

Confirming the VG Effect by Reversing Motion Phases. To further confirm the VG effect, we reversed the two motion phases (Figure 5A,B). Buffer A (0.5%MC, ×0.5TBE) and buffer B (0.5%MC, ×4TBE) were chosen for two motion phases. In the first system, motion phase I was buffer A and motion phase II was buffer B (A—B), $k = {}^1/_8$. DNA fragments moved at the speed of V_A in motion phase I and moved at the speed of ${}^1/_8V_A$ in motion phase II. The separation effect was anticipated to be poor because the VG effect was reversed. In the second system, the order of the buffer solutions was B—A, $k = 8$. DNA fragments moved at the speed of V_B in motion phase I and moved at the speed of $8V_B$ in motion phase II. The separation effect was anticipated to be better due to the VG effect. The results exactly confirmed our expectation. The B—A system showed a dramatically improved separation effect compared with A—B system. To avoid the effect of preconcentration on separation resolutions, the two-matrix-plug instead of three-matrix-plug in Figure 4 was adopted in the comparison. Given that two TBE buffer solutions showed similar separation capacity for DNA fragments (Figure 4B) and the sample peaks appeared in the time range of 130~350 s in both systems, the resolution-magnifying effect did not benefit from buffer concentration or running time.

It is worth noting that with increasing the k value, the peak heights of the DNA fragments also decreased. The possible reason was that the intercalated dye in the DNA fragments showed different fluorescence emission intensity in different BGS. We measured the fluorescence emission spectra of TO-PRO-3 and found that TO-PRO-3 had a tendency to emit weaker fluorescence in the lower concentration buffer (data not shown). This is the main reason why the preconcentration step was adopted in Figure 4. The preconcentration effect on separation resolutions could be omitted based on the results of Table 1. Also, the running time in MCE extended with the k value due to the increased resistance by filling the lower concentration buffer in the channel. Although we certified the VG theory by separating DNA fragments in microchip electrophoresis, the separation effect is not enhanced

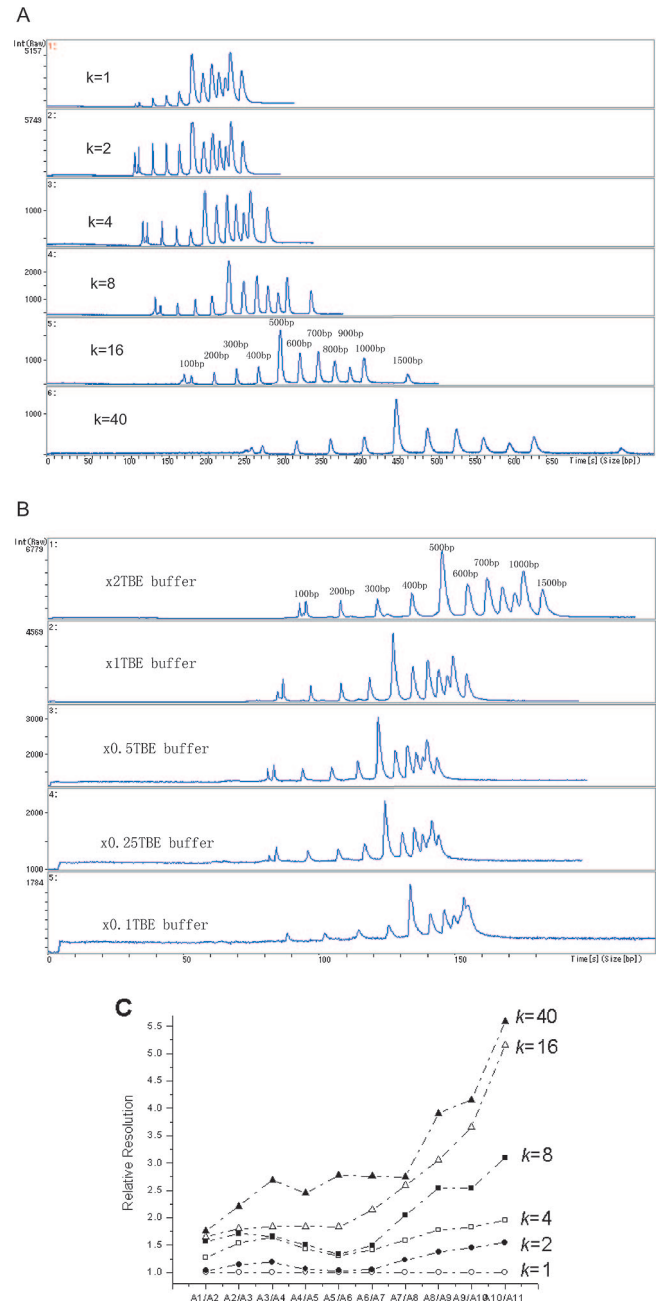

Figure 4. Practical application of VG theory in separating DNA fragments by MCE. k means C_1/C_2 (C_1 and C_2 are the buffer concentrations in motion phases I and II, respectively). Motion phase I is fixed with ×4TBE buffer. Motion phase II is substituted by ×4TBE ($k = 1$); ×2TBE ($k = 2$); ×1TBE ($k = 4$); ×0.5TBE ($k = 8$); ×0.25TBE ($k = 16$); ×0.1TBE buffer ($k = 40$), respectively. All buffer solutions contain 0.5%MC. The electropherograms of DNA fragments are shown in part A. The results of DNA separation in diluted TBE buffer are shown in part B. The relative resolutions are shown in part C, the absolute resolution of conventional separation ($k = 1$) was regarded as 1. Peaks A1—A11 represent 100bp—1500bp DNA fragments, respectively. The results confirm the resolution-magnifying effect of the VG theory in practical application. Separation conditions are described in the Experimental Section.

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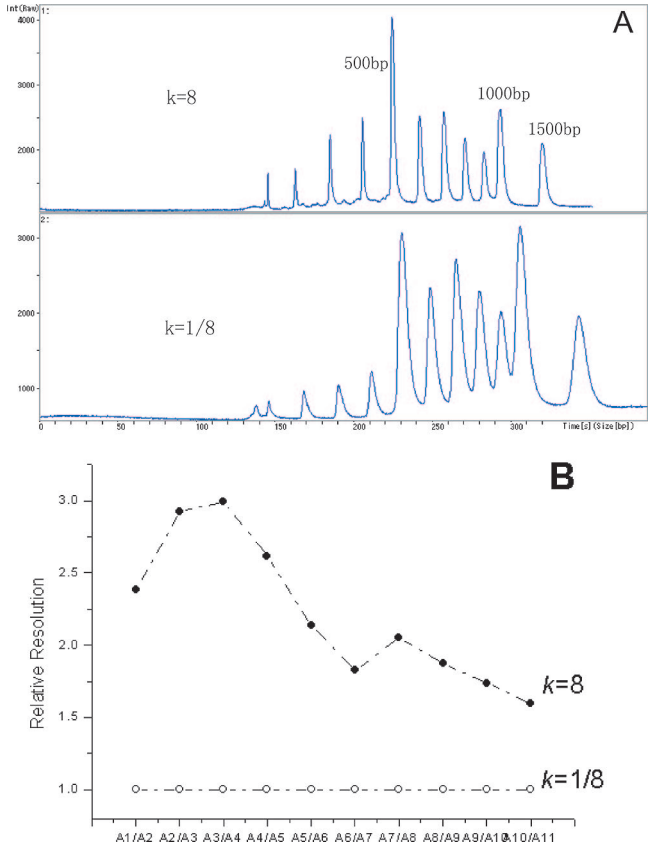

Figure 5. Confirming the VG effect by reversing the motion phases. In the system of $k = {}^1/_8$ (reversed VG effect), motion phase I is ×0.5TBE buffer and motion phase II is ×4TBE buffer. In the system of $k = 8$ (VG effect), motion phase I is ×4TBE buffer and motion phase II is ×0.5TBE buffer. All buffer solutions contain 0.5%MC. The separations are evaluated by electropherograms (A) and the relative resolutions (the absolute resolution of $k = {}^1/_8$ was regarded as 1) (C). The separation resolutions are dramatically enhanced by the VG effect compared with the reversed VG effect. For clear comparison, preconcentration step is omitted and two separation matrix plugs are adopted. Other separation conditions are described in the Experimental Section.

Table 1. Comparison of the Resolving Capacity with Two Injection Methods

	resolutions				
method	100/200bp	200/300bp	300/400bp	400/500bp	500/600bp
A[a]	9.96	6.27	4.50	3.31	2.29
B[b]	9.76	6.22	4.72	3.81	2.94

	resolutions				
method	600/700bp	700/800bp	800/900bp	900/1000bp	1000/1500bp
A[a]	1.78	1.27	1.06	0.83	1.74
B[b]	2.27	1.61	1.22	0.79	1.87

[a] Conditions of method A: injection buffer, ×4TBE; separation buffer, ×4TBE, MC0.5%; $E_{sep} = 168$ V/cm. [b] Conditions of method B: injection buffer, ×1TBE; separation buffer, ×4TBE, MC0.5%; $E_{sep} = 168$ V/cm.

as much as we theoretically confirmed. The main reason is that the VG effect could not be further improved due to ion diffusion. However, our results do confirm that the VG effect exists and could improve the separation effect. It is reasonable to speculate that a more efficient interface will overcome this disadvantage.

Potential Applications of the VG Theory. Several features indicate that the VG theory has diverse potential applications. (1) Since most separation techniques, such as chromatography and electrophoresis, utilize a velocity difference of mobilizing objects to separate analytes, the VG effect has a high compatibility with these techniques. Given that chromatography and electrophoresis are mainstays in many fields,[21−23] the VG effect may be potentially applied in a wide range. (2) The VG effect makes it possible to miniaturize the separation scale without losing the separation efficiency. Therefore, it has potential for microfluidic techniques to find "killer applications".[24] (3) By enlargement of the distance between two components, the VG effect could promote the purification efficiency which previously suffered a poor separation effect. (4) A high accuracy of genome sequencing is normally required by the low difference rate of human chromosomes (~1 of every 1 000 bases) for understanding the specific DNA variants[25] and by genomic research for disease biology.[26] The VG theory applied in separation possesses a great advantage to improve the sequencing accuracy by magnifying resolutions. (5) The VG theory in separation also has a potential application in finding disease biomarkers.[27]

CONCLUSIONS

A velocity gap theory was developed to magnify separation resolutions. Compared with a one-motion-phase of BGS in conventional separations, a two-motion-phase concept was proposed in VG separations. The VG effect occurred at an interface, so enhanced resolutions did not require extending separation lengths. The VG theory was theoretically confirmed by mathematical deduction. Also, its practical feasibility was certified by achieving high resolutions in MCE comparable with CE. It should be mentioned that the VG effect could not create a resolution, but it could magnify a resolution. In this regard, the VG effect is well compatible with other separation techniques, including chromatography and electrophoresis.

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