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Retardation Signal for Fluorescent Determination of Total Protein Content via Rapid and Sensitive Chip Moving Reaction Boundary Electrophoretic Titration

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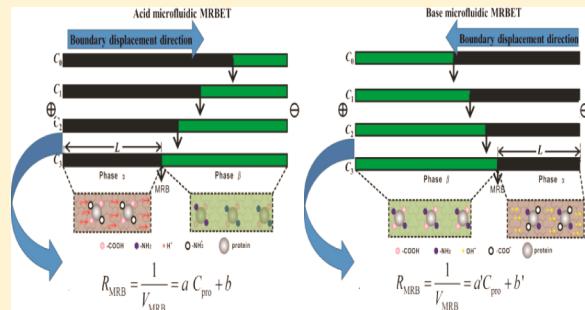
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Supporting Information

ABSTRACT: A novel concept and theory of moving reaction boundary (MRB) retardation signal (R_{MRB}) was advanced for determination of total protein content via MRB electrophoretic titration (MRBET). The theoretical results revealed that the retardation extent of boundary displacement, viz., the R_{MRB} value, was as a function of protein content. Thus, the R_{MRB} value of a sample could be used to determine its total protein content according to the relevant calibration curve. To demonstrate the concept and theoretical results, a novel microdevice was designed for the relevant experiments of MRBET. The microdevice has 30 identical work cells, each of which is composed of five ultrashort single microchannels (5 mm). In the microdevice, fluorescein isothiocyanate (FITC) was used to denote MRB motion and R_{MRB} value for the first time, the polyacrylamide gel (PAG) containing protein sample was photopolymerized in microchannels, and the MRB was created with acid or alkali and target protein sample. As compared to the classic Kjeldahl method and conventional MRBET performed in glass tube, the developed titration chip has the following merits: good sensitivity (0.3–0.4 $\mu\text{g}/\text{mL}$ vs 150–200 $\mu\text{g}/\text{mL}$ of protein concentration, 0.6–0.8 ng vs 30–2000 μg of absolute protein content), rapid analysis (20–60 s vs 15–200 min), and portable low-power (15 V vs 200 V).



Specific, rapid, and accurate determination of total protein contents in complex biological samples has been an urgent demand for clinical diagnosis,¹ proteomics,² and food science.³ There are a number of methods for the measurement of total protein contents, such as the Kjeldahl method,^{4,5} Dumas (N combustion) method,^{5,6} dye binding method,^{7–9} spectroscopy analysis method,^{10,11} and so on. However, there are still some issues in the protein content analyses, such as interference of non-protein nitrogen (NPN) reagents (e.g., melamine and urea), expensive instrumentation, and tedious procedures.^{5,12}

The technique of moving reaction boundary (MRB) offered an alternative tool for addressing these issues mentioned above. The concept of MRB was originated from the ideas of the “moving reaction front”,^{13,14} “precipitate reaction front”,^{15,16} and “stationary neutralization boundary”.¹⁷ In a MRB system,^{18,19} the positive reaction ion (e.g., hydrogen ion) and negative reaction ion (e.g., hydroxyl ion) are set in the anode and cathode vessels, respectively. Under an electric field, the two ions move in opposite directions, react with each other, and create a reaction boundary. On the basis of these concepts,

our group has developed systemic theories and methods of MRB for the improvement of isoelectric focusing (IEF),^{18,19} sample stacking of zwitterionic analytes,^{20,21} and sensitivity enhancement in monitoring of saccharide and glycoprotein.²²

Recently, the MRB electrophoretic titration (MRBET) has been presented as a rapid and accurate method for total protein measurement with anti-interference of NPN reagents.^{23,24} In a MRBET, the MRB was formed with an acid or base and target protein immobilized via continuous highly cross-linked polyacrylamide gel (PAG), and an acid–base indicator was used to denote the boundary location. The protein content of an unknown sample could be determined according to its calibration curve of boundary displacement versus protein content.^{23,24} However, the concept and theory of retardation signal have not been advanced for MRBET of protein content

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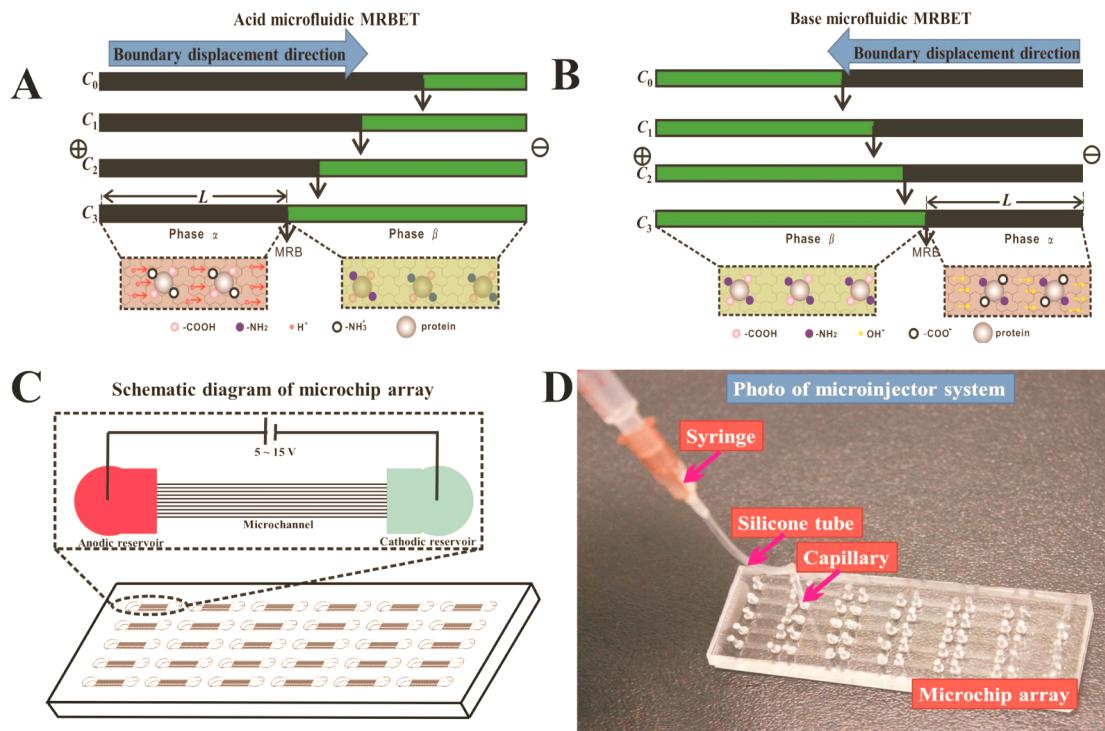


Figure 1. Diagrams of microfluidic MRBET. (A) Boundary motion (L) of MRB formed with acid and protein immobilized via PAG with fluorescent acid–base indicator. (B) Boundary motion (L) of MRB created with strong base and protein immobilized via PAG with fluorescent acid–base indicator. (C) Design of MRBET microdevice. (D) Photo of microinjector system for sample loading. In Panels A and B, $C_0 < C_1 < C_2 < C_3$; vertical arrows denote the boundary position; horizontal arrows indicate boundary displacement direction; the symbols “+” and “−” indicate the anode and the cathode, respectively. Other symbols are given in the figure and the context. In Panel D, microinjector is composed of syringe, silicone tube, and capillary.

measurement. Furthermore, the previous MRBET method performed in big glass tube device^{23,24} was of poor sensitivity and consuming time as well as a great quantity of sample consumption, greatly limiting its relevant application, especially in point-of-care testing characterized by short turn-around time, on-site availability, low cost, and disposability.

As one of the important electrophoretic properties, retardation signal is the extent to which analyte electromigration is retarded or delayed. For example, the electromigration of a protein molecule in pore limit electrophoresis was progressively retarded through a discontinuous cross-linked PAG, yielding a log–linear relationship between molecular weight and migration distance.^{25–27} The receptor mobility was retarded when a relevant affinity ligand was entrapped in the separating matrix of affinity electrophoresis. The extent of retardation of mobility was as a function of affinity ligand concentration. Thus, one could calculate the dissociation constants of affinity interaction systems (e.g., phosphorylase vs glycogen,²⁸ amylase vs starch,²⁹ and dextran-specific myeloma protein vs various dextrans³⁰). Unlike the two kinds of retardation signals, the boundary displacement in MRBET was decreased along with enhancement of protein content in PAG, as will be shown below. However, the retardation signal of MRB has not been advanced and investigated so far.

Microfluidic chips are considered as the ideal bioanalytical platforms with numerous merits of rapidity, low cost, high throughput, and good sensitivity.^{31,32} However, there are still some drawbacks in conventional microchip electrophoresis. For instance, a common microchip zone electrophoresis requires four ports to build a cross or double-T type injector, which needs a larger device footprint. Many efforts have sought to

simplify microfluidic electrophoretic system by scaling down channels and ports. For example, Ross et al. utilized a constant electroosmotic counter flow in conjunction with a modulated hydrodynamic counter flow for high-resolution separations in 3 mm length channels.³³ They used continuous sample introduction to form an injection zone, which required no extra channels or access ports.³³ Herr et al. demonstrated a protein electrophoresis platform relying on a single-inlet, single-outlet design and scaling down the channel length (1.3 mm) to enable low-power separation.^{34,35} However, there were still no chip, which could be directly used for the experiments of MRBET. In addition, the original acid–base indicator could not be used for the observation of boundary movement in chip due to its poor sensitivity. Hence, a fluorescent acid–base indicator ought to be introduced into chip MRBET for visualizing boundary displacement during the electrophoresis online and in real time.

Therefore, the main purposes of this paper are the following: (i) to propose the novel concept of retardation signal of MRB and relevant theory for determination of total protein content; (ii) to perform the proof-of-concept experiments for demonstrating the feasibility of developed theory via the simple MRBET-based microfluidic device with the sensitive acid–base indicator of fluorescein isothiocyanate (FITC). As a proof of principle, a set of model proteins and biosamples were chosen for the relevant experiments.

THEORY OF RETARDATION SIGNAL OF MRB

Figure 1A,B shows the schematic diagrams of MRB retardation signal (R_{MRB}). A protein sample is immobilized in single microchannels using highly cross-linked PAG (15% total

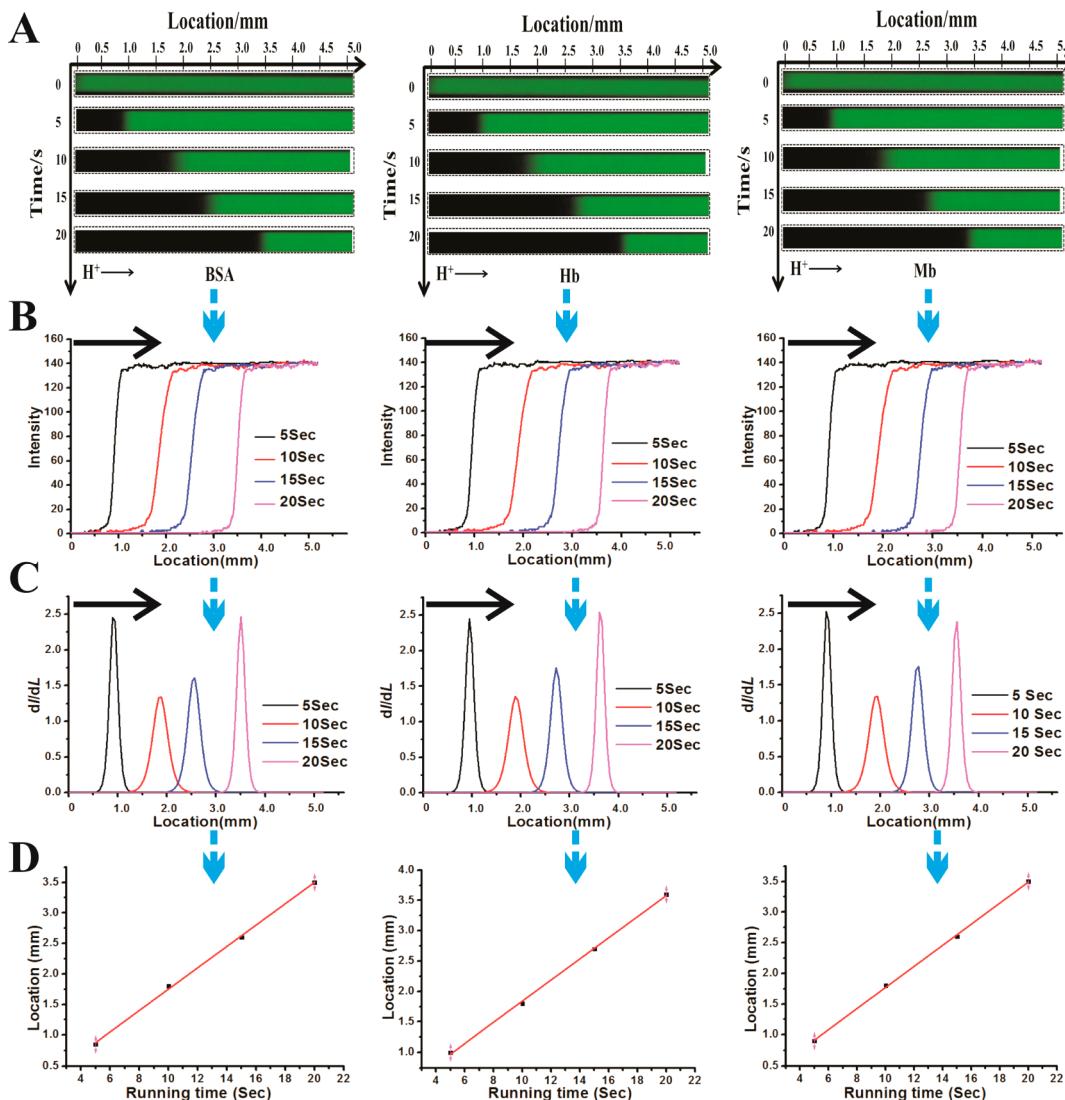


Figure 2. Experiments on acid microfluidic MRBET. (A) Photograph of boundary displacements during 0–20 s run of MRB created with 20 mM HCl and 2.0 mg/mL model proteins (BSA, Hb, and Mb). (B) Raw intensity data vs location obtained by line scanning along the microchannel axis in Panel A via MetaMorph software. (C) Derivatives of sigmoidal curves (dI/dL vs location) transformed from raw data in Panel B via Origin Pro 8.0 software. (D) Calibration curves of boundary motions in Panel C vs running time. Black arrows in Panels B and C indicate boundary displacement direction. Conditions: 100 mM KCl, 2 μ g/mL FITC, PAG (15%T and 4%C), 5 mm long microchannel (20 μ m deep \times 80 μ m wide), 30 V/cm and air-conditioned room (22–25 °C). Three measurements were made for each point.

acrylamide, T and 4% cross-linker, C). To conduct an electrophoretic acid titration (Figure 1A), the anodic reservoir is loaded with a strong acid (pH 2.30) and a platinum electrode is inserted into each well. In the base titration (Figure 1B), a strong base (pH 12.30) is set at the cathodic reservoir. The theory of R_{MRB} is detailed in the Supporting Information. The retardation signal of MRB (R_{MRB}) equation for protein titration is

$$R_{\text{MRB}} = aC_{\text{pro}} + b \quad (1)$$

where, C_{pro} is the concentration of protein, a and b are the two constants for protein titration (see the Supporting Information). Thus, the retardation signal can be defined as the ability of immobilized protein retarding MRB motion in protein titration. It ought to be noted that the MRBET is developed for determination of unknown concentration of known protein rather than an unknown protein concentration. Clearly, the

MRBET requires priori recognition of sample composition and establishes standards with the same sample composition.

EXPERIMENTAL SECTION

Reagents, protein samples, device fabrication and operation, on-chip gel photopolymerization, fluorescence imaging, and data analysis are all provided in the Supporting Information. Figure 1C shows the schematic diagram of MRBET microfluidic device. The compact microdevice has the size of a glass slide (7.5 \times 2.5 cm) with evenly distributed 30 (5 \times 6) cells. Each cell is composed of five single-inlet and single-outlet microchannels (20 μ m deep \times 80 μ m wide \times 5 mm long) powered solely with voltage control but without any pump, valve, and injector. The microchannels bridge the anodic reservoir and the cathodic reservoir (Figure 1C). The protein solution mixed uniformly with the gel solution was introduced into the channel using a custom-made microinjector system (Figure 1D).

Table 1. Linear Regression Equations between Boundary Location (L) and Running Time (t), and Linear Regression Equations between Retardation Signal of MRB (R_{MRB}) and Protein Content (C) in MRBET System

| sample | acid titration | | base titration | |
|--------|---------------------------------|---|----------------------------------|---|
| | L vs t^a | R_{MRB} vs C^b | L vs t^a | R_{MRB} vs C^b |
| BSA | $L = 0.175t$ $R = 0.999$ | $R_{\text{MRB}} = 0.05 + 0.0047C$ $R = 0.998$ | $L = 5.05 - 0.037t$ $R = 0.992$ | $R_{\text{MRB}} = 0.15 + 0.01C$ $R = 0.992$ |
| Hb | $L = 0.1 + 0.174t$ $R = 0.999$ | $R_{\text{MRB}} = 0.05 + 0.0032C$ $R = 0.999$ | $L = -0.8 + 0.055t$ $R = 0.995$ | $R_{\text{MRB}} = 0.143 + 0.06C$ $R = 0.998$ |
| Mb | $L = 0.05 + 0.172t$ $R = 0.999$ | $R_{\text{MRB}} = 0.05 + 0.0045C$ $R = 0.998$ | $L = -0.95 + 0.057t$ $R = 0.997$ | $R_{\text{MRB}} = 0.144 + 0.006C$ $R = 0.990$ |
| milk | $L = 0.094t$ $R = 0.999$ | $R_{\text{MRB}} = 0.058 + 0.024C$ $R = 0.98$ | $L = -0.2 + 0.033t$ $R = 0.997$ | $R_{\text{MRB}} = 0.178 + 0.057C$ $R = 0.98$ |

^aThe linearity equations correspond with those in Figures 2, S-3, S-7, and S-8 (Supporting Information). ^bThe linearity equations correspond with those in Figures 3, 4, S-4, and S-9 (Supporting Information).

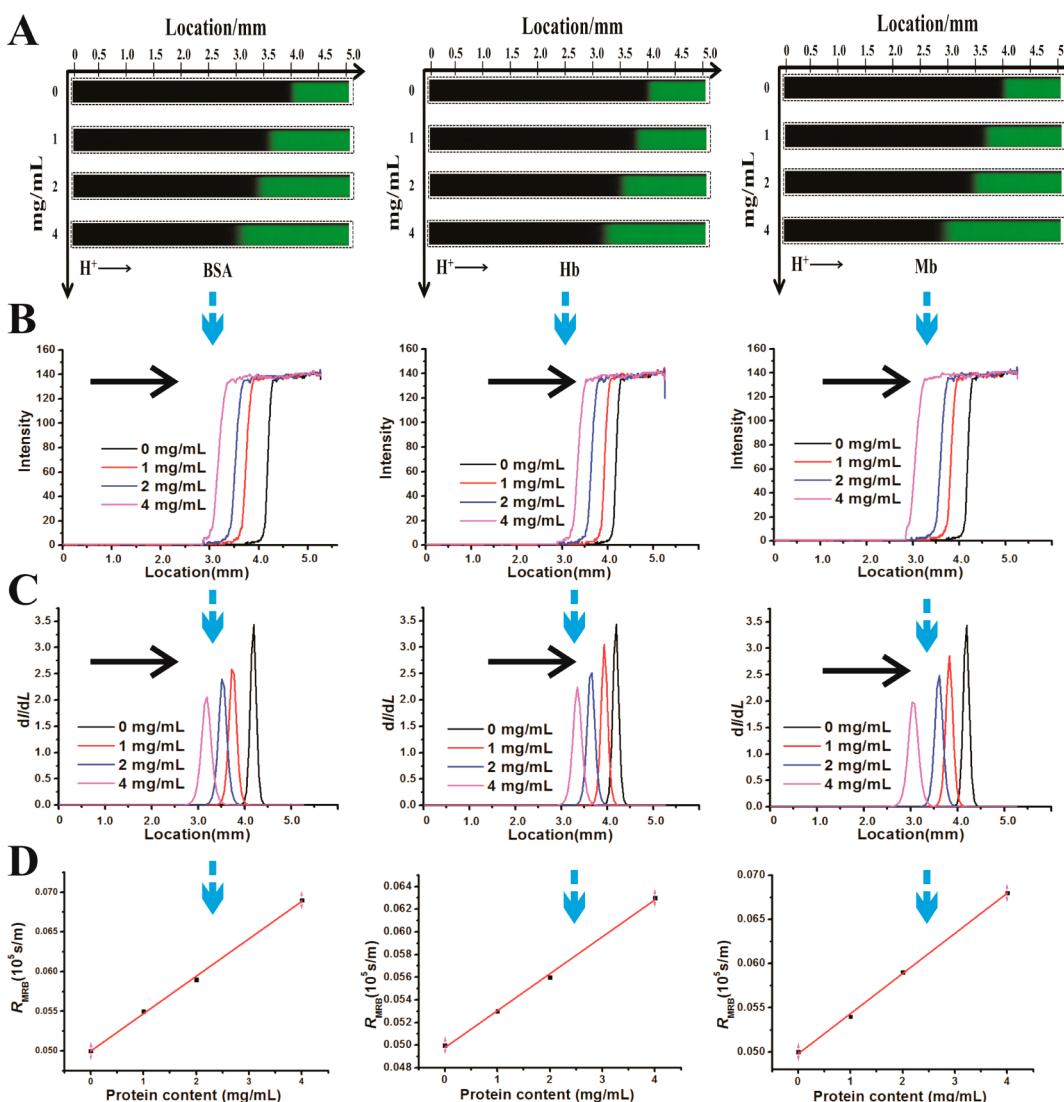


Figure 3. Experiments on acid microfluidic MRBET. (A) Photograph of boundary motions at 20 s run of MRB created with 20 mM HCl and three model proteins (BSA, Hb, and Mb) with different concentrations (0, 1, 2, and 4 mg/mL). (B) Raw intensity data vs location in Panel A. (C) Derivatives of sigmoidal curves (dI/dL vs location) transformed from raw data in Panel B. (D) Calibration curves of retardation signal of MRB (R_{MRB}) calculated from Panel C vs protein content. The black arrows in Panels B and C indicate boundary direction. The other experimental conditions are the same as those in Figure 2. Three measurements were made for each point.

RESULTS AND DISCUSSION

Various optical acid–base indicators (e.g., phenolphthalein and bromophenol blue) have been used to detect the proton and hydroxide ion electromigration,^{36,37} or monitor MRB displacement in a big glass tube.^{23,24} However, the optical indicators cannot denote MRB displacement in microfluidic system due to its poor sensitivity. FITC was widely used in the fluorescent

tracing of antibody and rapid diagnosis of some diseases in medical fields.^{38,39} The pH chemical sensor had been investigated based on the fluorescence property of FITC.⁴⁰ Herein, FITC was used as a fluorescent acid–base indicator denoting MRB displacement in a single microchannel based on the relationship between the fluorescence intensity of FITC and pH values. A series of gel solutions of pH 2.24–13.9 were

prepared with 1.0 M hydrogen chloride and 1.0 M sodium hydroxide. The effect of pH on the fluorescence intensity of FITC (2 $\mu\text{g}/\text{mL}$) in a single microchannel of a MRBET device was investigated, and the relationship between the fluorescence intensity and pH is shown in Figure S-2 (Supporting Information). As can be seen in Figure S-2 (Supporting Information), the fluorescence intensity of FITC increased with a rise in pH value in the range of pH 2.24–9.6, then decreased in the range of pH 10.77–13.9. Obviously, FITC could be used as a fluorescent acid–base indicator to monitor the pH value of either acidic or basic solutions. In addition, FITC could weakly react with proteins under the given conditions due to the low pH, rapid preparation of gel, and high ratio of protein to FITC in the MRBET. Thus, FITC could be well used for denoting MRB displacement in real time in a microfluidic chip, as demonstrated in the following experiments.

To demonstrate microfluidic MRBET, three model proteins of BSA, Hb, and Mb were chosen for the relevant experiments. In the acid microfluidic MRBET, 20 mM HCl and 2 $\mu\text{g}/\text{mL}$ FITC (w/v) were used as the strong acid and the indicator, respectively. Figure 2 showed the acid microfluidic MRBET experiments on the displacements of MRB created with hydrogen ions and protein samples (BSA, Mb, and Hb of 2 mg/mL) during the runs from 0 to 20 s. As shown in Figure 2A, all boundaries moved toward the cathode because of high flux of hydrogen ions from the anode electrolyte. It was observed that the whole single microchannel presented the green fluorescence of FITC before each run. However, when electric field was applied, the anodic side of microchannel became dark because of fluorescence quenching by the hydrogen ions electromigrating from the anode solution toward the cathode. As the run continued, the dark zone became longer and longer. The results in Figure 2A directly demonstrated there was a stable MRB displacement.

However, the boundary was unclear for naked eyes. Thus, the images in Figure 2A were transformed as raw intensity data versus boundary displacement by line scanning along the microchannel axis (see Figure 2B). Then the data of raw intensity were transformed as the derivatives of sigmoidal curves wherein the maximum was used to establish where the change in response was greatest per unit time (Figure 2C). From the data in Figure 2C, the distances of boundary displacement in different running time could be easily obtained. The fitting curves in Figure 2D depicted the relationship between the boundary displacement and the running time, and the relevant correlation coefficient was $R > 0.99$ (also see Table 1), indicating a good linearity. All of the results in Figure 2 manifested that the boundary displacement was proportional to the running time of acid microfluidic MRBET.

The experiments were further performed on the retardation signal versus protein content. Figure 3A revealed the boundary location in single microchannel at 20 s run of MRB created with 20 mM HCl and three model proteins at different concentration levels (0–4 mg/mL). The boundary moved slowly if the protein concentration was increased, qualitatively indicating the validity of eq 1. The experimental retardation signals could be simply computed via eq S-10 (Supporting Information). Figure 3B shows the raw intensity data versus location in Figure 3A, and Figure 3C displays the derivatives of sigmoidal curves (dI/dL vs location) transformed from the raw data in Figure 3B. Figure 3D displayed the relation between retardation signal and protein content, and the high coefficient ($R > 0.99$). Furthermore, the linearities with different slopes

existed between retardation signal and protein content for different protein samples due to various numbers of alkaline groups per protein molecule (Figure 3D and Table 1). According to the theory of R_{MRB} , these linear equations should have the same intercept if ion mobilities were invariant in MRBET. Actually, the linear equations with a little different intercepts were listed in Table 1. This deviation may be systematic or random. The ion mobilities were influenced by the temperature, ionic strength, gel property, and protein.^{23,24}

The base microfluidic MRBET was also systematically investigated in the Supporting Information. As compared with the boundary displacements in the acid titration analyses in Figures 2 and 3, these in the base titration in Figures S-3 and S-4 (Supporting Information) were generally slower than the ones in the acid titration under the given conditions. The fact reflected very different electromigration behaviors of hydrogen and hydroxy ions in the gel with immobilized proteins. The migration behaviors were very complex and will be discussed in our further study. Meanwhile, the moving boundaries observed in the base MRBET runs were not as clear and stable as the ones in the acid MRBET. The reason is still unknown at the present time.

To investigate the specificity of microfluidic MRBET, we also conducted the parallel titration runs for milk samples added with glucose, melamine, Gly, and NAD at three concentration levels of 0.01, 0.1, and 0.2 mg/mL. Figures S-5 and S-6 (Supporting Information) show that the measured R_{MRB} values of samples added with different compounds were very similar to each other, indicating that the added reagents would not change the R_{MRB} values. These results suggested that the microfluidic MRBET had a strong ability against the influence of melamine adulterated in protein samples. In contrast, the Kjeldahl method or Dumas method could not differentiate protein nitrogen from some NPN reagents (e.g., melamine), leading to false increases in the protein content of adulterated milk and food products.^{5,12}

The limit of detection (LOD) values of acid and base microfluidic MRBET runs based on 3σ of the variability of the displacement in the case of a “blank” sample were 0.3 $\mu\text{g}/\text{mL}$ (0.6 ng of absolute protein content) and 0.4 $\mu\text{g}/\text{mL}$ (0.8 ng of absolute protein content), respectively. The LOD value of concentration in the microfluidic MRBET decreased about 500-fold as compared with the one from the Kjeldahl method (200 $\mu\text{g}/\text{mL}$)⁵ and the one from conventional MRBET performed in a glass tube (150 $\mu\text{g}/\text{mL}$).²³ The LOD value of absolute protein content in microfluidic MRBET reduced about 2.5×10^6 -fold as compared with the one from the Kjeldahl method (2000 μg)⁵ and about 3.75×10^4 -fold as compared with the one from the conventional MRBET performed in a glass tube (30 μg).²³

Precision of microfluidic MRBET was evaluated by measuring relative standard deviations (RSD) of boundary location from 5.0 to 20 s for acid titration and from 20 to 80 s for base titration in one microchannel. Using BSA of 2.0 mg/mL as a model, the RSD values for three parallel measurements were 3.4% for acid microfluidic titration and 5.8% for base titration, indicating a good precision.

Recovery experiments were designed to evaluate the accuracy of microfluidic MRBET. The recovery was calculated by the ratio of the protein content detected via microfluidic MRBET in one microchannel to the precise content measured by an electronic balance. The recoveries were more than 96% for BSA, more than 95% for Hb, and more than 92% for Mb in acid

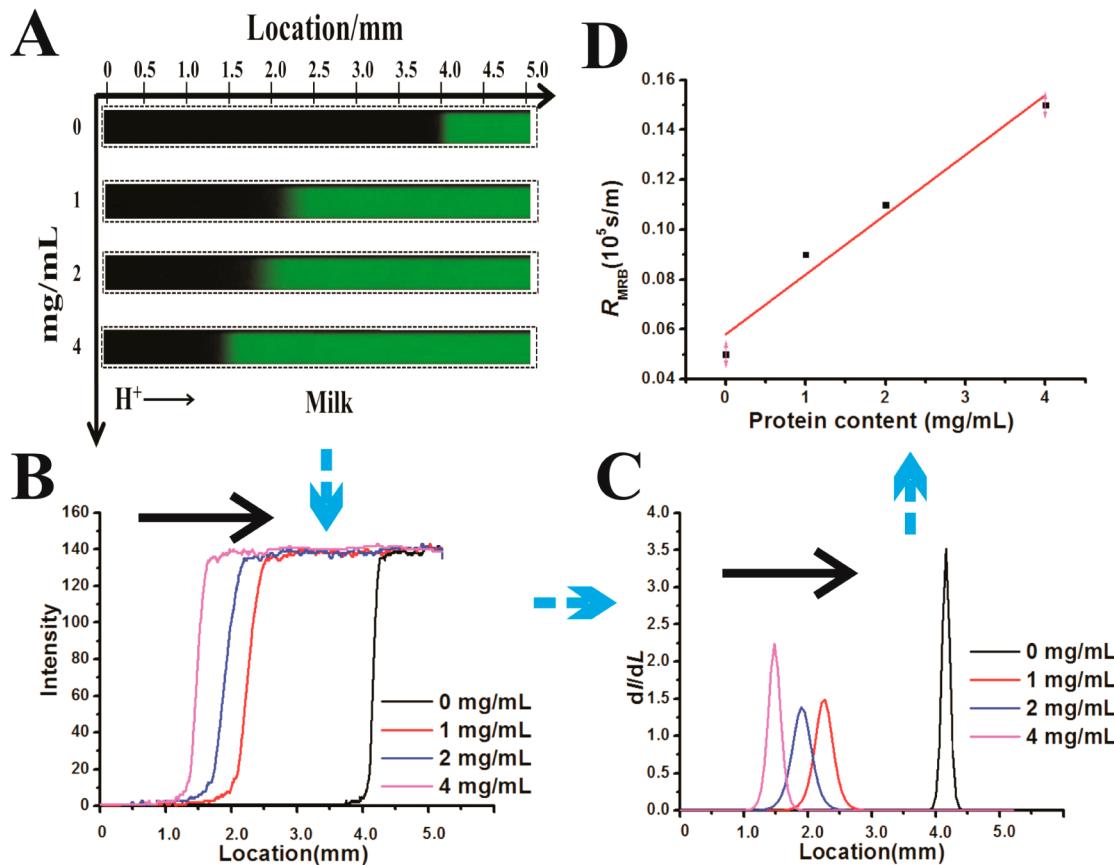


Figure 4. Experiments on acid microfluidic MRBET. (A) Photograph of boundary locations at 20 s run of MRB created with 20 mM HCl and different protein content (0, 1, 2, and 4 mg/mL) infant milk powder sample. (B) Raw intensity data vs location in Panel A. (C) Derivative of sigmoidal curve (dI/dL vs location) transformed from raw data in Panel B. (D) Calibration curve of R_{MRB} calculated from Panel C vs protein content. Black arrows in Panels B and C indicate boundary displacement direction. Other conditions are the same as those in Figure 2. Three measurements were made for each point.

titration. Accordingly, they were more than 94% for BSA, more than 97% for Hb, and more than 93% for Mb in base titration. This result suggested that the proposed method had a fairly good accuracy.

As compared with the Kjeldahl method^{4,5} or conventional MRBET method,^{23,24} the developed microfluidic MRBET has the following features. First, the content of protein with known composition could be quickly determined within around 20 s in acid titration, or 60 s in base titration according to relevant linear regression equations (Table 1), greatly faster than that of the Kjeldahl method (more than 3 h)^{4,5} and conventional MRBET method (about 15 min).^{23,24} Second, a low sample volume (2 μ L) was required in the developed method as compared with that of the Kjeldahl method (10–20 mL)^{4,5} and MRBET method (200 μ L).^{23,24} Third, MRB displacement in microfluidic MRBET was controlled by a low applied voltage (15 V), and saved much power in contrast to the MRBET method (200 V),^{23,24} which could be applicable to portable low power assays.

To demonstrate the feasibility of the developed method in analyzing real biological sample, we chose infant milk powder solution for acid and base electrophoretic titration. In microfluidic MRBET, the establishment of calibration curve of milk powder was assisted by the Kjeldahl method. As shown in Figure S-7 (Supporting Information), distance of boundary motion L was proportional to the running time t of MRB created with hydrogen ions and milk protein, and there was a

good linear relation between R_{MRB} and total protein content ($R > 0.98$), as shown in Figure 4. Similarly, the good linearities were also observed in the base titration (Figures S-8 and S-9 in the Supporting Information).

The linear regression equation of total protein concentration of unknown milk samples measured by acid titration and base titration was $C_{\text{acid}} = -0.012 + 1.01C_{\text{base}}$ ($R = 0.997$), manifesting that the good correlation between acid and base microfluidic titrations. In addition, the values of protein content in the milk sample measured by the developed method got close to the ones obtained via the Kjeldahl method. The linear regression equation was $C_{\text{acid, MRBET}} = 0.08 + 0.96 C_{\text{Kjeldahl}}$ ($R = 0.998$), suggesting that the total protein content in the milk sample detected via microfluidic MRBET were in good agreement with those achieved with the classic Kjeldahl method. These experimental results further demonstrated the validity of the developed method.

As compared with the original equation of MRB (viz., eq S-2, Supporting Information), the equation of retardation signal (viz., eq 1) is very straightforward and could be used with effortlessness for determination of protein content, as has been demonstrated above. Comparing with the common photoelectric signal, electrochemical signal and affinity retardation signal,^{28–30} the developed MRB retardation signal has the following characteristics. First, according to the linearity of eq 1, the retardation signal of boundary displacement is as a function of protein content in electrophoretic titration process. Second,

the retardation signal could be easily obtained via experimental eqs S-1 and S-9 (Supporting Information), indicating the retardation signal is only associated with the relative displacement of boundary and weakly influenced by other factors (e.g., indicators, gels). The weak impact of an indicator on electrophoretic titration has been demonstrated and discussed in detail in the previous reports.^{23,24,41} Third, the retardation signal possesses high stability, tolerating many nonprotein substances. In addition, the retardation signal could also be used in other MRB models, such as transient MRB electrohoresis²¹ and moving supermolecule boundary electrophoresis.²²

CONCLUSIONS

From the results and discussion mentioned above, one can conclude that a novel theory and method of R_{MRB} were first advanced for determination of total protein content. Second, a compact microdevice was designed for the relevant experiments of MRBET, and FITC was used to denote MRB displacement and R_{MRB} in real time. Third, the systemic experiments revealed that R_{MRB} of boundary displacement was a function of protein content, demonstrating the validity of developed theory and method. Thus, the retardation signal could be used for determination of protein content of known sample. In addition, the developed method exhibited the merits of low limit of detection, high speed, good reproducibility, and low power consumption.

ASSOCIATED CONTENT

Supporting Information

Text describing the theory of retardation signal of MRB, reagents and protein samples, Kjeldahl procedures, device fabrication and operation, step-by-step process of on chip gel photopolymerization with schematic diagram, weak influence of 15S electrode reaction, fluorescence imaging and data analysis, and demonstration of base microfluidic MRBET. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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