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Controlled Protein Separation based on Pressure-Voltage (P-V) Coupling Effects in a Nanopore based Device

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Abstract

The separation and/or purification of biomolecules are of great importance in biotechnological analysis, characterization and related application. In this work, a set of "U"-type device containing a feed cell and a permeation cell connected by nanopore arrays was designed for controlled protein separation. Certain voltage (as driving force) and pressure difference (△P, as resistance) were applied to make selected protein to move from the feed cell into the permeation cell through nanopore arrays. The resultant of driving forces applied to proteins can be modulated by changing the parameters such as applied voltage, pH value and △P between two cells, thus their translocation velocity of can be controlled. By finding the P-V equilibrium points for Bovine serum albumin （BSA）and hemoglobin from bovine （Hb）, the separation ratios (mass ratio) for BSA and Hb can be achieved as BSA : Hb=12.5 and Hb : BSA=14.3 receptively.

Keywords: nanopore; protein separation; voltage; pressure

1. Introduction

The separation and/or purification of biomolecules are of great importance in biotechnological analysis, characterization and application, especially in *in vitro* analysis, antibody generation, binding assays and structural studies which require pure protein samples with different sizes, shapes, charges, and hydrophobicities [1-10]. Generally, ultrafiltration [11], electrophoretic separation [12], liquid chromatography, magnetic nanoparticles based separation [13] and membrane chromatography [14-15] are common methods for biomolecules separation or purification, among which membrane separation and ultrafiltration have been extensively adapted [16-19]. In membrane based separation, it requires critical structures and properties of membrane, especially for definite pore sizes. However, the general use of membranes with a broad distribution of pore size and heterogeneous matrices leads to low protein adsorption capacities. From another point of view, if separation or purification can be determined by other factors out of pore size, thus more precisely controlled separations may be expected.

In recent years, nanopore or nanopore arrays based analytical technology provides potentials to fabricate novel nanofluidic or microfluidic device for biomolecule sensing and separation [20-27]. The general picture of nanopore or nanopore arrays based nanofluidic device can be illustrated as followings: a pair of separated liquid cells with certain electrolyte solutions is linked by a chip containing a nanopore or nanopore arrays; along the length direction of nanopore certain voltage is applied, charged biomolecules and ions can migrated from one cell to another through

nanopores by electrophoresis. If other factors are introduced to nanopore based device, the movements of biomolecules can be controlled more precisely. For example, Golovchenko and his coworkers [28] showed that the threading and detection functions could be decoupled by the addition of a pressure bias across a voltage-biased solid-state pore. In a pressure-voltage (P-V)-biased pore, the net force (and hence the speed) of a biomolecule could be reduced by an order of magnitude without a similar reduction in the ionic current through nanopores. The movement of biomolecule depends on an experimentally adjustable balance of the applied pressure and voltage gradients.

These also provide novel ideals for biomolecules separation. In this work, a set of "U"-type device containing a feed cell and a permeation cell connected by nanopore arrays was designed and employed for controlled protein separation. The voltage and pressure difference (△P) between two liquid cells can be changed in a certain range, thus protein separation can be achieved based on this device via a pressure-voltage (P-V) coupling control.

2. Experimental

2.1 Materials

Polycarbonate membranes containing nanopore arrays (pore diameter: 50nm, pore density: 6 pores/μm^2) were purchased from Whatman, Inc. Bovine serum albumin (BSA, pI=4.9) and hemoglobin from bovine (Hb, pI=7.0) [29] were obtained from Shi Feng Inc. Ultra-pure water (resistivity 18.25 MΩ.cm) was used for all the solutions and rinsing.

2.2 Device

A "U"-type device containing a feed cell (on the right side) and a permeation cell (on the left side) was designed and employed in our experiments, as showed in **Fig.1**. Two separated cells with certain solutions were linked by a piece of PC membrane containing nanopore arrays and they were sealed by PDMS. In our experiment, electric field was applied to make proteins in the feed cell to transport to the permeate cell through nanopore arrays, and the values of △P generated by vacuum pump were adjusted in order to produce an resistant force. The direction of the electric field will be adjusted to guarantee the electric field force serving as the driving force: when protein was positively charged, the anode and the cathode were set in the feed cell and the permeate cell respectively; when protein was negatively charged, the electrodes were reversed.

2.3 Parameters for Pressure-Voltage (P-V) Coupling experiments

BSA and Hb were employed in our work for protein separation experiments. The applied voltage was 1.0 V to 3.0V, and the pH value for main experiments was controlled at 7.4 or 4.0, and the ion strength was controlled at 0.105 mol/L. △P was controlled at 0 MPa, 0.02 MPa, 0.04 MPa, 0.06 MPa and 0.08 MPa respectively. All of the experiments were carried out at room temperature, and the time for individual protein translocation or protein separation is 3 hours. The concentration of protein in the feed solution was 1 μM (66400 μg/mL for BSA, 65000 μg/mL for Hb), the concentration of protein in the permeation cell which reflected the separation efficiency was determined by the strength of UV-vis absorbance. The characteristic

UV-vis wavelengths were at 291 nm and 405 nm for Hb, 291 nm for BSA respectively. In our experiments, the concentration for Hb can be determined as C_{Hb}=241.23483*A_{405nm}+4.4, and the concentration for BSA can be determined as C_{BSA}=6922.81006*$A_{BSA291nm}$-44.91. (In the last equation, $A_{BSA291nm}$=A_{291nm}-$A_{Hb291nm}$= A_{291nm}-0.24861*A_{405nm}+0.00179. Here C_{Hb} (μg/mL) and C_{BSA} (μg/mL) stand for the calculated concentration of Hb and BSA respectively; A_{405nm}, A_{291nm}, $A_{Hb291nm}$, $A_{BSA291nm}$ stand for the total absorbance at 405nm, the total absorbance at 291nm, the absorbance at 291nm contributed by Hb and the absorbance at 291nm contributed by BSA respectively.

3. Results and discussion

3.1 Pressure-Voltage (P-V) Coupling effects

The basic idea for controlled protein separation based on pressure-voltage (P-V) coupling effects can be illustrated as **Fig.2.** For protein A, it will transfer from the feed cell into the permeation cell under the driving of voltage; at the same time, △P can generate a resistance for its translocation. If the force generated by △P is bigger than that generated by voltage, protein A could transfer back into the feed cell theoretically. That is to say, there must be a P-V equilibrium point (EP_A) for protein A, at which the driving force is equal to the resistance. Similarly, there must be a P-V equilibrium point (EP_B) for protein B. Generally, the conformation and charge state of the proteins are different from each other, so EP_A is not the same with EP_B. At EP_A, the main movements of protein A is free diffusion because of the equilibrium of driving force and resistance, while protein B can move from the feed cell into the

permeation cell under the residual driving force (the resultant of forces generated by voltage and △P). By this way, the separation of protein A and protein B can be achieved by pressure-voltage (P-V) coupling effects. Of course, finding out the P-V equilibrium point of the single protein is the basis for the separation of proteins by pressure-voltage (P-V) coupling effects.

Fig.3 gives the pressure modulation in the translocation process of BSA and Hb through nanopore arrays with time lapse. The black and green points stand for proteins translocation only under the driving of voltage, while the red and blue points stand for proteins translocation under the synergic driving of voltage and ΔP. The concentration in the permeation cell can be determined by the changes in UV-vis spectra of the solution, as showed in the inserted image ① in **Fig.3**. Here the translocation ratio (the concentration ratio of the two proteins obtained from individual translocation experiments, BSA : Hb or Hb : BSA) is defined to evaluate the relative translocation abilities of BSA and Hb. Based on the data in **Fig.3**, the translocation ratio (BSA : Hb) at different time can be calculated, as showed in the inserted image ② in **Fig.3**. Under the driving of voltage or synergic driving of voltage and △P, the protein concentrations in the permeation cell increase with time lapse approximate linearly, which indicated their migration rates are near a constant under given conditions. Obviously, the migration rates for both BSA and Hb are decreased under the resistance from △P of 0.04 MPa, which indicates protein's translocation can be modulated by the comprehensive control of voltage and △P. In addition, the translocation ratio (BSA : Hb) seems to increase with time lapse,

especially for the case under the comprehensive control of voltage of 3.0 V and △P of 0.04 MPa.

3.2 Protein translocations

Fig.4 shows the individual voltage-driven translocation behaviors of BSA and Hb modulated by △P when the pH value of the solution is 7.4 or 4.0, and the total time for each experiment are 3 hours. Here the applied voltage (3.0 V or 1.5 V) works as a driving force and △P works as a resistance. With △P increasing, the average translocation velocities of BSA and Hb are both decreased, no matter that the voltage are 3.0 V and 1.5 V (as showed in the inserted picture of **Fig.4a** and **Fig.4b**). At the same time, it can be found that the translocation abilities of BSA and Hb are different from each other. According to the experimental results showed in **Fig.4a** and **Fig.4b**, the translocation ratios of BSA to Hb (BSA : Hb) obtained under the driving voltage of 3.0 V are bigger than those obtained under the driving voltage of 1.5 V. With △P increasing, the translocation ratios of BSA to Hb (BSA : Hb) obtained under the voltage of 3.0 V continue to increase, while those obtained under the voltage of 1.5 V become to decrease after reaching the maximum value at about △P=0.04 MPa. According to these data, The biggest translocation ratio of BSA to Hb (BSA : Hb) is 23.75 when the pH value, applied voltage and △P are controlled at 7.4, 3.0V and 0.08 MPa respectively.

As reported in former literatures, changing pH value can change the charged amounts of proteins, which will alter the electrostatic force applied to proteins. Definitely, the alternation in pH value of the solution will change the voltage-driven

translation behaviors of proteins. **Fig.4c** and **Fig.4d** show the individual translocation behaviors of BSA and Hb modulated by △P when the pH value of the solution are 7.4 and 4.0 respectively, and the total time for each experiment are 3 hours. The applied voltage of 3.0 V works as a driving force and △P works as a resistance. Obviously, net charges belonging to these two proteins are changed because of pH value changing. Similarly, with △P increasing, the average translocation velocity of BSA and Hb are decreased, no matter that the pH value are 4.0 and 7.4. With △P increasing, the translocation ratios of Hb to BSA (Hb : BSA) obtained in the solution with the pH value of 4.0 continue to increase, while those obtained in the solution with the pH value of 7.4 are almost unchanged. In our experiment, the biggest translocation ratio of Hb to BSA (Hb : BSA) is 8.0 when the pH value, applied voltage and △P are controlled at 4.0, 3.0V and 0.08 MPa respectively.

3.3 Proteins separations

In separation experiments, two kinds of proteins (BSA and Hb, their initial concentrations in the feed cell were both 1 μM) were added in the feed cell of the device. Here the separation ratio (the concentration ratio of the two proteins obtained from mixture translocation experiments, BSA : Hb or Hb : BSA) is defined to evaluate the relative separation abilities. **Fig.5** gives the separation ratio via △P increasing, which is similar to the translocation ratio changing tendency showed in **Fig.4**. The separation ratios of BSA to Hb (BSA : Hb) obtained under the driving voltage of 3.0 V are bigger than those obtained under the driving voltage of 1.5 V. Similarly, with △P increasing, the separation ratios of BSA to Hb (BSA : Hb)

obtained under the driving voltage of 3.0 V continues to increase, while those obtained under the driving voltage of 1.5 V become to decrease after reaching the maximum value at △P=0.04 MPa. The biggest separation ratio of BSA to Hb (BSA : Hb) is 12.5 when the pH value, voltage and △P are controlled at 7.4, 3.0 V and 0.08 MPa respectively. When pH value of the solution is 7.4, both proteins are negatively charged. However, because BSA has a lower isoelectric point, the charge belonging to BSA is bigger than the charge belonging to Hb. This point (pH=7.4, voltage=3.0 V, △P=0.08 MPa) can be regarded as an approximate P-V equilibrium point for Hb (EP_{Hb}), at which Hb keeps staying in the feed cell due to the balance of driving force and resistance while BSA can migrate from the feed cell into the permeation cell. In this case, P-V equilibrium point for Hb (EP_{Hb}) is the basis for the separation. PH value, voltage and △P are three important factors for P-V equilibrium. If one of them is changed, the equilibrium will be destroyed; but new equilibrium can be reached by changing the other one or two factors. **Fig.5** indicates that △P for the best separation value increases from 0.02 MPa to 0.08 MPa with the driving voltage increasing from 1.0 V to 3.0 V. For Hb (EP_{Hb}), the voltage increasing means the larger driving force, and it needs bigger △P for the next P-V equilibrium, which is the reason that the △P corresponding to the best separation shifts to bigger value with the driving voltage increasing. For BSA, it is always in a nonequilibrium state (driving force is always bigger than resistance). The increment in driving force generated by voltage increasing is bigger than the resistance increment generated by △P, which results in the increasing tendency of the best separation (from 6.3 to 12.5, as showed in the

inserted picture in **Fig.5**) value with the driving voltage varying from 1.0 V to 3.0 V.

On the other hand, pH value can determine net charges on the protein and thus influence the driving force generated by voltage. Definitely, the alternation in pH value of the solution will change the P-V equilibrium points of proteins thus change the parameters for the proteins separation based on pressure-coupling effects. In the following experiment, pH value of the solution is decreased to 4.0, at which BSA and Hb are both positively charged and the net charges belonging to Hb is bigger than the net charges belonging to BSA. As showed in the inserted picture in **Fig.6**, when △P is less than 0.06 MPa, BSA concentration in the permeation cell is bigger than Hb concentration. This maybe attribute to the shape of BSA which is more advantageous to diffuse than Hb, and the electrophoretic contribution is not big enough to change the diffusion influence of BSA, which has been mentioned by Chun and Strovev [30]. When △P are 0.06 MPa and 0.08 MPa, the concentrations of Hb in the permeation cell became bigger than BSA. With △P increasing, the resultant of forces applied to BSA became smaller and smaller, the amount of BSA transporting across nanopores decreased greatly, while Hb can still transport into the permeation cell because of relative bigger electrical force. The biggest separation ratio of Hb to BSA (Hb : BSA) is 14.3 when the pH value, applied voltage and △P are controlled at 4.0, 3.0V and 0.08 MPa respectively. Similarly, this point (pH=4.0, voltage=3.V, △P=0.08 MPa) can be regarded as an approximate P-V equilibrium point for BSA (EP_{BSA}), at which BSA keeps staying in the feed cell due to the balance of driving force and resistance while Hb can migrate from the feed cell into the permeation cell. Similarly, if pH is

changed now, the equilibrium will be destroyed, and related separation ratio will be also changed, as showed in the inserted picture of **Fig.6**. Taking into account their isoelectric points, if the pH value of the solution is between 4.0 and 7.4, the two proteins will be differently charged (positive and negative), and the separation by pressure-voltage (P-V) coupling effects will be difficult to achieve.

Conclusion

In summary, we provide a new way to achieve proteins separation based on pressure-voltage (P-V) coupling effects in a nanopore based fluidic device, which provide a new possibility and choice for protein selection or separation. The resultant of driving forces applied to proteins can be modulated by changing the parameters such as voltage, pH value and △P between two cells, thus their translocation velocity of can be controlled. By finding the P-V equilibrium points for BSA and Hb, the separation ratios (mass ratio) for BSA and Hb can be achieved as BSA : Hb=12.5 and Hb : BSA=14.3 receptively.

It should be pointed out that the separation speed will be reduced under these equilibrium points. However, the separation efficiency can be improved by optimizing the experimental apparatus and method (for example, to find new P-V equilibrium point under smaller value of ΔP; to enlarge the effective working areas of polycarbonate membranes to provide more nanopores for protein translocation). Better parameters for more efficient separation are now in progress.

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Figure captions

Figure 1： "U"-type reaction device containing a feed cell and a permeation cell. The pressure difference (△P) and the voltage between the two cells can be modulated in order to achieve pressure-voltage (P-V) coupling effect based separation of proteins.

Figure 2： The illustration for the mechanism of proteins separation based on pressure-voltage (P-V) coupling effect.

Figure 3： The translocation process of BSA and Hb through nanopore arrays with time lapse: the black and blue points stand for proteins translocation under the driven of voltage, while the red and green points stand for proteins translocation under the driven of voltage pressure-voltage (P-V) coupling effect. The concentration in the permeation cell can be determined by the UV-vis spectra of the solution from the cell. **Inserted image ①:** As an example, the UV-vis spectra for Hb translocation at different time are showed in the inserted picture).

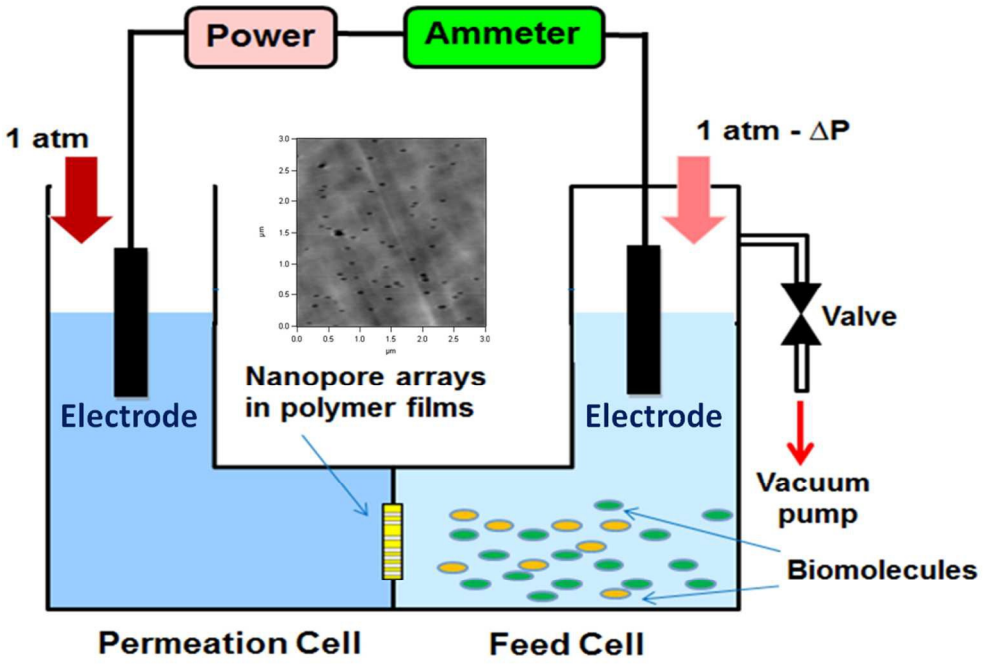
Inserted image ②: the translocation ratio (BSA : Hb)

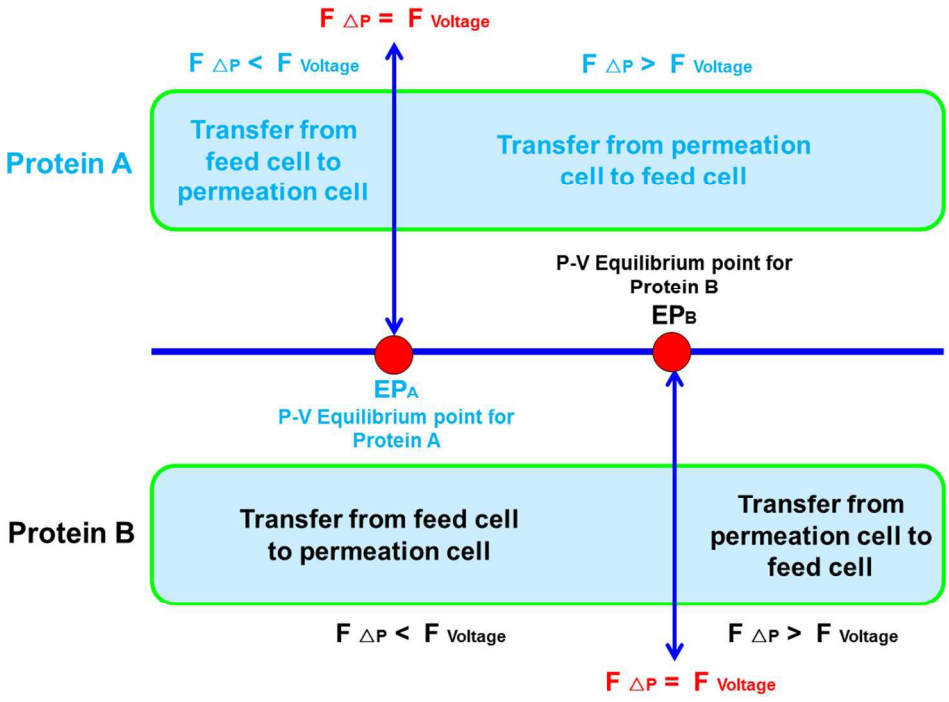
Figure 4： The translocation ratio for BSA and Hb (the blue line and point stand for BSA : Hb; the red line and point stand for Hb: BSA) obtained from independent translocation. (a) 3.0 V, pH=7.4; (b) 1.5 V, pH=7.4; (c) 3.0 V, pH=4.0; (d) 3.0 V, pH=7.4;

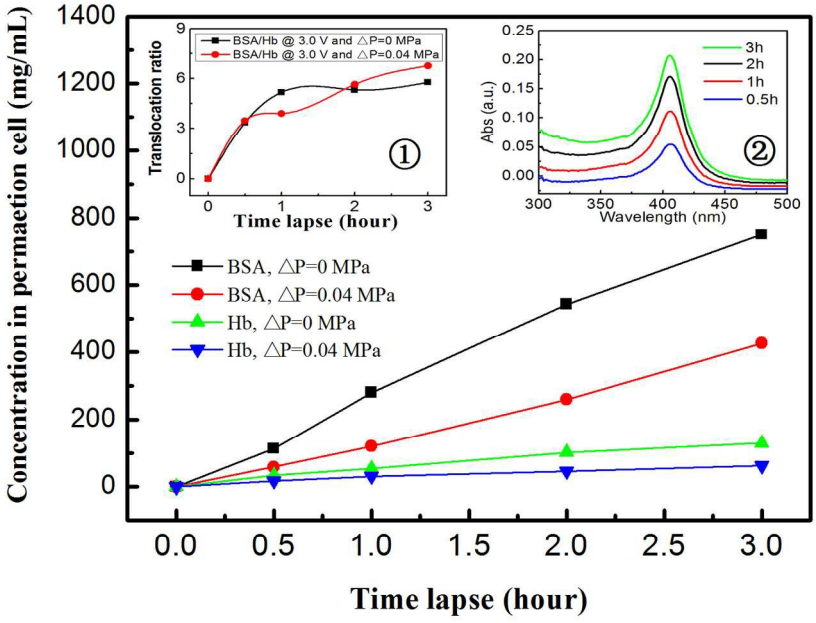
The inserted picture showed the concentrations of BSA and Hb in the permeation cell after a 3-hour experimental time. The driven voltage was controlled at 3.0 V or 1.5 V, and the pH value was controlled at 7.4 or 4.0

Figure 5： The separation ratio for BSA and Hb (BSA : Hb) obtained from mixed translocation under different driving voltage after a 3-hour experimental time.
The inserted picture showed ΔP corresponding to the best separation and the value of the best separation under different driving voltage. The pH value was controlled at 7.4. The driven voltage was changed from 1.0 V to 3.0 V.

Figure 6： The separation ratio for Hb and BSA (Hb : BSA) obtained from mixed translocation after a 3-hour experimental time. The driven voltage was controlled at 3.0 V, and the pH value was controlled at 7.4 or 4.0.
The inserted picture showed the separation ratio (SR) of Hb : BSA if pH value was controlled at 7.4, 9.0, 10.0 or 4.0, 3.0, 2.0 repectively.







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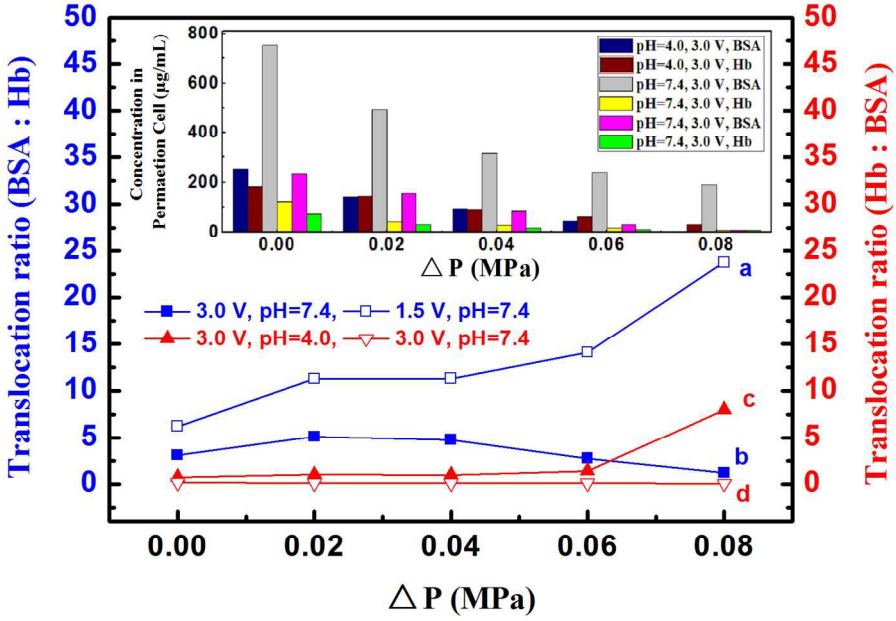

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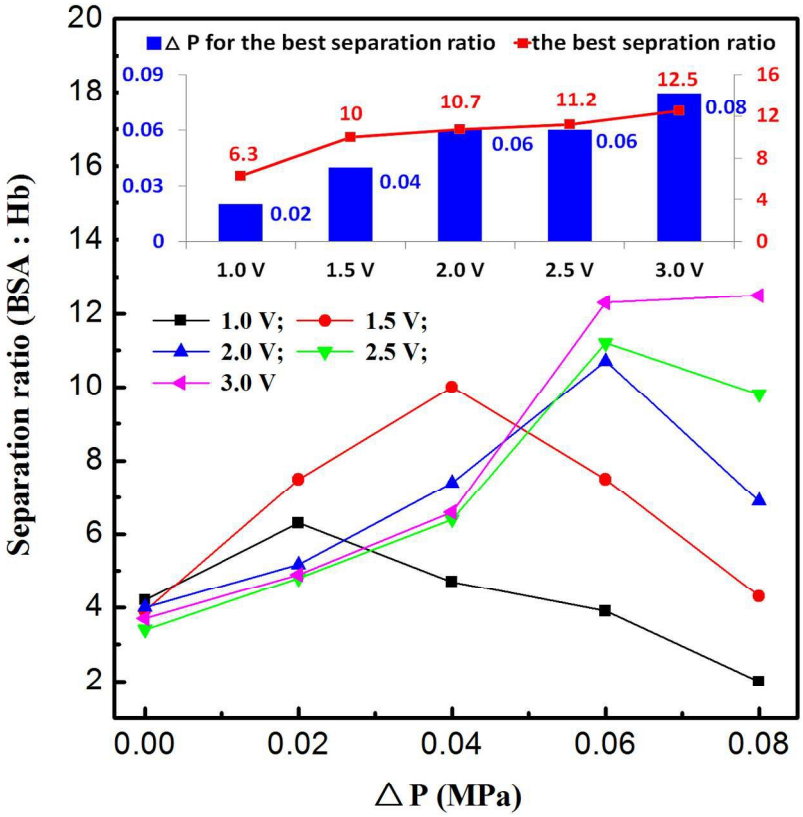

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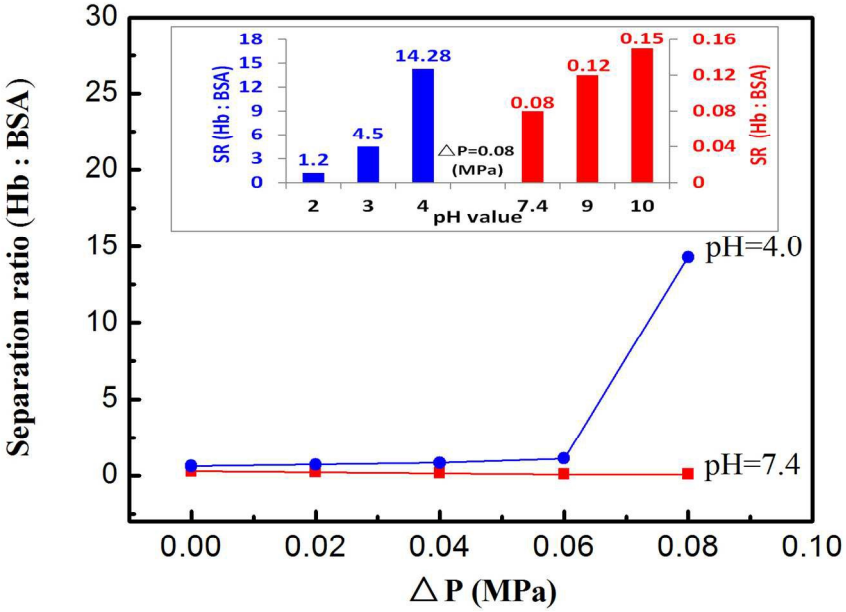

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