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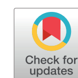
Construction of chiral ligand exchange capillary electrochromatography for D,L-amino acids enantioseparation and its application in glutaminase kinetics study☆



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ABSTRACT

A chiral ligand exchange capillary electrochromatography (CLE-CEC) protocol was designed and implemented for D,L-amino acids enantioseparation with poly(maleic anhydride-styrene-methacryloyl-L-arginine methyl ester) as the coating. The block copolymer was synthesized through the reversible addition fragmentation chain transfer reaction. In the constructed CLE-CEC system, poly (methacryloyl-L-arginine methyl ester) moiety of the block copolymer played the role as the immobilized chiral ligand and Zn (II) was used as the central ion. Key factors, including pH of buffer solution, ratio of Zn (II) to ligands, the mass ratio of monomers in the block copolymer, which affect the enantioresolution were investigated. Comparing with the bare capillary, the CLE-CEC enantioresolution was enhanced greatly with the coating one. 5 Pairs of D,L-amino acids enantiomers obtained baseline separation with 5 pairs partly separated. The mechanism of enhancement enantioresolution of the developed CLE-CEC system was explored briefly. Further, good linearities were achieved in the range of 25.0 μM–5.0 mM for quantitative analysis of D-glutamine (r^2 = 0.997) and L-glutamine (r^2 = 0.991). Moreover, the proposed CLE-CEC assay was successfully applied in the kinetics study of glutaminase by using L-glutamine as the substrate.
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1. Introduction

Chirality is one of the most important characteristics of nature or life process, and chiral compounds are widely used in many areas [1]. Most of the compounds that construct the basic units of life are chiral molecules, thus different spatial configurations between the enantiomers have a significant influence on the composition of the life system and biological activity [2–5]. Amino acids (AAs), the basic units of proteins and peptides with two different D,L configurations, play a vital role in many life activities. However, there is a significant difference between D-AAs and L-AAs in biological activities. For example, L-AAs are the important nutrients in human bodies, which widely used in food and pharmaceutical areas, while D-AAs are related to physiological processes [6,7]. Moreover, some D,L-AAs related enzymes have displayed their catalytic specificities on different configurations of AAs. For example, glutaminase, an amidohydrolase enzyme, which has been used as an efficient anti-retroviral agent in treating human immunodeficiency virus therapy, catalyses L-glutamine (L-Gln) to L-glutamic acid (L-Glu) [8,9]. Therefore, analysis and separation of D,L-AAs is of great significance.

In recent years, varieties of analytical methods have been developed for D,L-AAs enantioseparation analysis [10,11]. Among these numerous methods, chiral ligand exchange capillary electrophore-

Abbreviations: P(MAn-St-MAA), poly(maleic anhydride-styrene-methacryloyl-L-arginine methyl ester); CLE-CEC, chiral ligand exchange capillary electrochromatography.

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sis, with metal complex as chiral selectors, has exhibited much advantages in its simplicity and controllable enantiomer migration order [12,13]. However, these chiral metal complex based systems also meet the challenges of limited ligands and narrow applications [14]. Nowadays, chiral ligand exchange capillary electrochromatography (CLE-CEC) with immobilized ligands has attracted much interest [15]. In CLE-CEC, the ligands with chiral recognition capability can be immobilized onto the inner surface of the capillary or packed into the capillary. Subsequently, the D,L-AAs enantiomers could be well separated based on the ligand exchange principle with the immobilized or packed ligands [16,17].

Usually, L-AAs, D-AAs, their derivatives and some of chiral organic acids are used as the chiral ligands in D,L-AAs enantiomers analysis [18]. Several of these ligands have been immobilized onto the capillaries to construct novel CLE-CEC systems for realizing well enantioseparations of D,L-AAs [19–27]. For example, Chen and colleagues [26] made a L-phenylalaninamide-modified monolithic silica column and developed a CLE-CEC method to separate Dns-D,L-AAs with copper acetate and 70% acetonitrile in the buffer solution. Aydoğan and co-workers [27] prepared a CLE-CEC system by modifying L-histidine onto the capillary inner surface to realize the chiral separation with copper sulfate and 60% acetonitrile in the running buffer. However, some of the assays suffered from complicated operation process and time consuming [28]. Moreover, much organic solvent in running buffer is unfriendly to separate biological samples. Therefore, it is desirable and important to develop novel CLE-CEC systems with distinctive immobilized chiral ligands and without organic solvents in buffer solution for enantioseparation of D,L-AAs.

In this study, a block copolymer, poly(maleic anhydride-styrene-methacryloyl-L-arginine methyl ester) (P(MAn-St-MAA)), was first prepared and chemically bonded onto the inner wall of the capillary. Then a new open-tubular CLE-CEC system was constructed for D,L-AAs enantioseparation with PMAA moiety of the block copolymer as the immobilized chiral ligand and with Zn (II) as the central metal ion. We demonstrated that by using the proposed CLE-CEC method, well enantioseparation of D,L-AAs could be successfully achieved without organic solvents in running buffer. The possible chiral recognition mechanism was explored. Furthermore, the developed CLE-CEC assay was applied in glutaminase kinetics study with L-Gln as the substrate, showing the feasibility of the protocol in practical application.

2. Materials and methods

2.1. Materials and chemicals

The reagents for P(MAn-St-MAA) synthesis included styrene (St) from Chemical Plant (Shanghai, China), maleic anhydride (MAn) from Guangfu reagent (Tianjin, China), and L-arginine methyl ester dihydrochloride from Sigma-Aldrich (St. Louis, USA). The initiator azobisisobutyronitrile (AIBN) and transfer agent Benzyl benzodithioate (BBDT) was bought from Shanghai Chemical Plant (Shanghai, China) and Sigma-Aldrich (St. Louis, USA), respectively. 3-Aminopropyltriethoxysilane (APTES) and thiourea were obtained from Aladdin Reagent Co Ltd. (Shanghai, China).

D,L-amino acid (D,L-AA) enantiomers, dansyl chloride (Dns-Cl), were obtained from Sigma-Aldrich Chemical Co. (St. Louis, USA). Glutaminase (from Escherichia coli) was provided by Megazyme International Ireland Ltd. (Wicklow, Ireland).

Sodium hydroxide, ammonium hydroxide, zinc sulfate ($ZnSO_4$), tris (hydroxymethyl) amino-methane (Tris), lithium carbonate, boric acid, sodium acetate, dimethyl sulfoxide (DMSO), *N*, *N*-dimethylformamide (DMF), 1,4-dioxane, ethanol, tetrahydrofuran (THF), diethyl ether and other reagents were all provided by Beijing Chemical Corporation (Beijing, China). L-arginine (L-Arg) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Milli Q (Millipore Co., Massachusetts U.S.A) water was used for the preparation of all the solutions.

2.2. Apparatus

All of the Dns-D,L-AAs enantioseparations and the glutaminase kinetics study were carried out on a high performance capillary electrophoresis analyzer with a UV detector at 254 nm (Beijing Institute of New Technology and Application, Beijing, China). The 100 μm and 75 μm i.d. × 60 cm (45 cm effective) capillaries (Yongnian Optical Fiber Factory, Hebei, China) were used for separation experiments.

2.3. Solution preparation

All of solutions were prepared with triply distilled water and stored at 4 °C. Standard D,L-AAs stock solutions (2.0 mg/mL) were diluted by $10–10^4$ fold for quantitative analysis. Dns-D,L-AAs were derived according to the reference [14]. Briefly, 20.0 μL D,L -AAs, 20.0 μL 40.0 mM lithium carbonate and 20.0 μL Dns-Cl (1.5 mg/mL in acetone) were added in a 200 μL vial and kept at microwave irradiation (480 W) conditions for 6.0 min. The CLE-CEC running buffer (pH 8.0, adjusted by Tris) was composed by 100.0 mM boric acid, 10.0 mM ammonium acetate, 4.0 mM L-Arg and 4.0 mM $ZnSO_4$. It was filtrated with 0.45 μm membrane filter, and degassed by sonication for 2 min before use.

2.4. Synthesis of P(MAn-St-MAA)

Monomer, methacryloyl-L-arginine methyl ester (MAA), was synthesized by acylation reaction according to the literature [29]. Specifically, 3.7 g (14.2 mM) L-arginine methyl ester dihydrochloride and 2.7 mL triethylamine were added into 75.0 mL dichloromethane, and after the mixture was totally dissolved, added 1.4 mL (14.3 mM) methacryloyl chloride under ice bath with stirring. Then the above mixture was stirred at room temperature for 24 h. Finally the product was precipitated with excess ether, filtered and dried in the vacuum oven at 50 °C.

The macromolecular transfer agent P(MAn-St) was synthesized through the reversible addition fragmentation chain transfer (RAFT) reaction with the initiator AIBN and transfer reagent BBDT. The reaction took place in a 3-neck flask with the nitrogen protection. The molar ratio between St, MAn, AIBN, BBDT was 1000:100:0.2:1. St was 10.0 mM with 5.0 mL 1,4-dioxane, magnetic stirring at 60 °C for 24 h. Then the product was precipitated with excess ether, filtered and dried in the vacuum oven at 50 °C.

The synthesized macromolecular transfer agent P(St-MAn) and MAA monomer were utilized for P(MAn-St-MAA) synthesis with DMF (10.0 mL) as the solvent, AIBN (10.0 mg) as the initiator. Then the solution was degassed by freeze-evacuate-thaw cycles in nitrogen atmosphere and reacted for 24 h at 60 °C. Finally the product was precipitated with excess water, filtered and dried in the vacuum oven at 50 °C. Polymer-1, polymer-2 and polymer-3 represented different mass ratio between P(MAn-St) and MAA (Table S1), and the synthesis processes were same as described above.

2.5. Coated capillary and CLE-CEC analysis

Firstly, the bare capillary was rinsed with 1.0 M NaOH for 3 h, and then water and methanol for 0.5 h, respectively. After dried for 1 h, APTES and THF (50/50, v/v), subsequently the P(MAn-St-MAA) solution dissolved in DMSO (15 mg/mL) were used to rinse the capillary for 12 h. Finally, the capillary was dried for 24 h for further use.

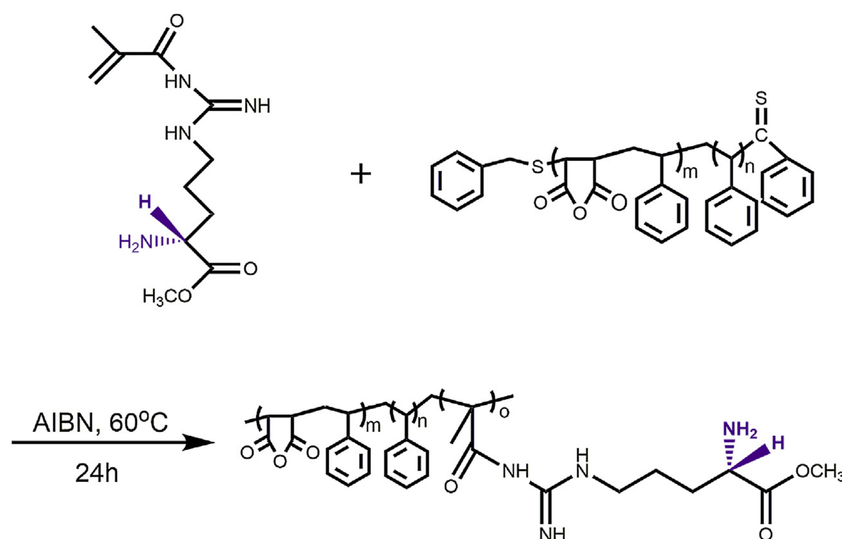

Fig. 1. Schematic of synthesis process of P(MAn-St-MAA).

Unless stated otherwise, the CLE-CEC running buffer was composed of 100.0 mM boric acid, 10.0 mM ammonium acetate, 4.0 mM zinc sulfate, and 4.0 mM L-Arg at pH 8.0. The applied separation voltage was at −20 KV. Samples were siphoned to the capillary for 8 s at 15 cm height and detected at anode side through UV detector at 254 nm. Thiourea was used as the marker for measurement of electroosmotic flow (EOF).

2.6. Kinetics study of glutaminase

The kinetics constants of glutaminase were determined by the constructed CLE-CEC system. L-Gln dissolved in a buffer solution (40.0 mM sodium acetate, adjusted to pH at 4.9 with 36% acetic acid (v/v)) was used as the substrate of the glutaminase. All enzymatic reactions were performed in 0.2 mL polypropylene tubes at 37 °C. The final concentration of glutaminase was 5.0 μg/mL. 200 μL L-Gln and 200 μL enzyme solution were mixed together and incubated at 37 °C for 1 min. Then the reaction was terminated by being heated in boiling water for 10 min, and then centrifuged at 10,000 rpm for 10 min. Finally, the supernatants were collected, derived, and applied to CLE-CEC. The kinetics study of glutaminase was initiated by adding L-Gln solution of various concentrations (0.5 mM, 0.9 mM, 1.5 mM, 3.0 mM and 6.0 mM) into the glutaminase solution. The kinetics of the glutaminase was evaluated by Michaelis-Menten's constant (K_m) and maximum rate (V_{max}). Three replicates (n = 3) were measured for each concentration value.

3. Result and discussion

3.1. Synthesis and characterization of P(MAn-St-MAA)

The block copolymer, P(MAn-St-MAA), was selected as the capillary coating for enantioseperation of D,L-AAs with the proposed CLE-CEC system. MAA monomer was prepared through the acylation reaction (Fig. S1), and then reacted with the macromolecular transfer agent P(MAn-St) through RAFT reaction to obtain P(MAn-St-MAA), and the synthesis process was shown in Fig. 1.

Fourier transform infrared (FT-IR) spectra of MAA and P(MAn-St-MAA) was exhibited in Fig. S2. C=O in carboxyl of arginine at 1662 cm^{-1} could be observed in Fig. S1a. As exhibited in Fig. S1b, except the C=O of arginine in MAA, CH_2- in benzene ring at 2927 cm^{-1} and C=C in benzene ring at 1541 cm^{-1} and 1495 cm^{-1} were observed, which indicated the benzene ring in the synthesized polymer. The typical peak C=O in maleic anhydride could be observed at 1780 cm^{-1} and 1854 cm^{-1}. The results showed that block copolymer, P(MAn-St-MAA), was synthesized successfully.

3.2. P(MAn-St-MAA) coating characterization

The coating process of P(MAn-St-MAA) was displayed in Fig. S3. The anhydride bond of MAn in P(MAn-St) moiety of the block copolymer could chemically bond with amino group of the silylated capillary [30] and PSt moiety of the polymer could provide a uniform coating on the capillary inner wall. While, PMAA moiety could stretch into the buffer solution and be used as the immobilized chiral ligand to realize the CLE-CEC enantiomers separation.

Scanning electron microscope (SEM) images of the bare capillary and coated capillary were depicted in Fig. 2. The surface of the bare capillary was smooth while a rough surface in coated capillary was observed, which indicating the block copolymer was bonded on the inner surface of the capillary successfully.

3.3. Repeatability and stability of the coating capillaries

The stability of the polymer-2 coated capillary was evaluated by measurement of EOF in continuously runs. The relative standard deviation (RSD) of 30 consecutive runs EOF measurement was 4.6%, which showed good stability of the block copolymer coating. The electrochromatogram of EOF marker was exhibited in Fig. S4, and the EOF value was calculated as $(0.556 \pm 0.02) \times 10^{-4}\ cm^2\ v^{-1}\ s^{-1}$. The capillary-to-capillary and intra-capillary RSDs of migration times and peak areas were both less than 5.0% (Table S2), indicating a good repeatability.

3.4. Enantioseparation of Dns-D,L-AAs

By constructing a new CLE-CEC system with P(MAA) moiety of the block copolymer as the immobilized chiral ligands and Zn(II) as the central ion, the separation of Dns-D,L-AAs was investigated. Key factors of CLE-CEC conditions, such as: buffer pH, concentrations of central ions and mass ratio of monomers in the block copolymer, effect on enantioresolution were optimized in detail. Three pairs of Dns-D,L-AAs, including D,L-serine (D,L-Ser), D,L-methionine (D,L-Met) and D,L-Gln, were selected as the test analytes. It should be mentioned that L-Gln was selected because it could be used as the

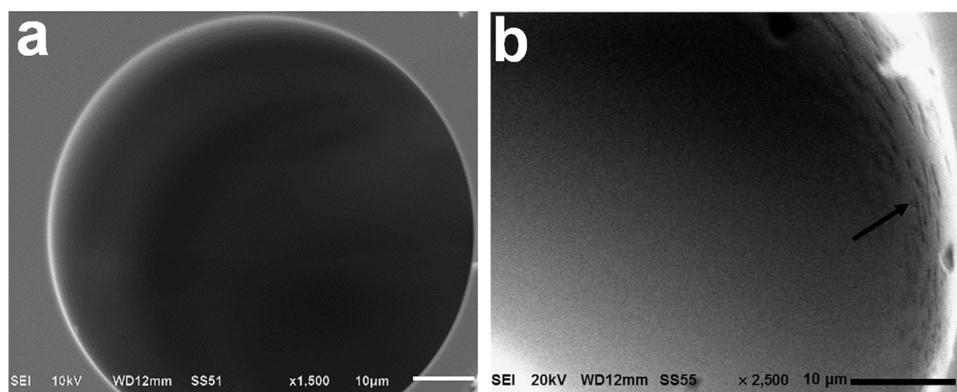

Fig. 2. SEM images of bare capillary (a) and coated capillary (b).

substrate of glutaminase, D,L-Ser and D,L-Met were chosen for their particularities with hydroxyl group and thiol group, respectively.

As we know, pH of running buffer is a key factor on enantioresolution. Fig. 3A and B shows that the migration time and chiral resolution (R*s*) increased with pH increasing at range from 7.1 to 8.1, respectively. When the buffer pH was lower than 7.9, the test three pairs of Dns-D,L-AAs couldn't obtain baseline separation. At pH 8.1, the migration time of the three test analytes was prolonged greatly. In order to get the highest R*s* within shorter migration time, pH at 8.0 was selected for the following research.

The concentration ratio of Zn (II) to L-Arg in buffer solution is another key factor for influence the enantioresolution in CLE-CEC. Fig. 3C and D exhibits that the test Dns-D,L-AAs couldn't achieve baseline separation when the concentration ratio of Zn (II) to L-Arg in buffer solution was 5:1–2:1. Once the ratio of Zn (II) to L-Arg was equal to 1:1 or 1:2 or 1:3, the test Dns-D,L-AAs could be baseline separated with the migration time was prolonged obviously at 1:1 or 1:2, while shortened at 1:3. Taking consideration of R*s* and migration time, the ratio of Zn (II) to L-Arg at 1:1 was chosen for further study.

The next crucial factor for affect the enantioresolution in CLE-CEC is the concentration of Zn(II). Fig. 3E and F depicts the investigation results of the concentration of Zn(II) ranging from 2.0 mM to 7.0 mM. We observed that the test Dns-D,L-AAs could be baseline separated at 3.0 mM or 4.0 mM Zn(II). While, their migration times decreased with the increasing of the concentration of Zn(II). For obtaining a higher resolution within shorter migration time, 4.0 mM Zn(II) was selected finally.

In the constructed CLE-CEC system, except L-Arg as free ligand in buffer solution, the chiral recognition of the immobilized ligand in the block copolymer coatings indeed played a vital role in D,L-AAs enantiomers separation. Therefore, the effect of mass ratio of monomers in block copolymer on the enantioresolution in CLE-CEC was investigated. Here, using P(MAn-St) and MAA as the monomers, three different block copolymers were synthesized, and the results were exhibited in Fig. S5. It could be found that when block copolymer-1 (mass ratio of P(MAn-St):MAA was 2:1) was used as the capillary coating, although shorter migration times were obtained, Dns-D,L-Gln and Dns-D,L-Met couldn't achieve the baseline separation. Interestingly, the enantioresolution of the three test analytes were greatly improved when block copolymer-2 (mass ratio of P(MAn-St):MAA was 1:1) was coated onto the inner wall of the capillary with the prolonged migration times. While, shorter migration times and decreased R*s* were observed when block copolymer-3 (mass ratio of P(MAn-St):MAA was 1:1.5) was used as the capillary coating. Obviously, block copolymer-2 was selected finally as the coating one for constructing the CLE-CEC system. It should be noted that the polymer molecular weight value of the block copolymer-2 determined by gel permeation chromatography (GPC) was 18.9 kD and its polydispersity index was 1.4.

Also, wider coated capillary with i.d. 100 μm was used to investigate the influence of inner diameter of the capillary on the enantioresolution. The results exhibited in Table S3 showed that the three test Dns-D,L-AAs couldn't be baseline separated, the R*s* was much lower than the data obtained with the i.d. 75 μm coated capillary (Table S4). Thus, 75 μm was selected finally.

With the block copolymer-2 as the capillary coating and P(MAA) as the immobilized chiral ligand, 17 pairs Dns-D,L-AAs were enantioseparated by the proposed CLE-CEC system under the optimized CLE-CEC conditions: 100.0 mM boric acid, 10.0 mM ammonium acetate, 4.0 mM Zn(II), 4.0 mM L-Arg at pH 8.0. Moreover, the proposed CLE-CEC method showed a good robustness as displayed in Fig. S6. Finally, 5 pairs of D,L-AAs enantiomers were attained baseline separation, and 5 pairs were partly separated as depicted in Fig. 4 and Table S4. It should be noted although 7 pairs of D,L-AAs enantiomers could not be enantioseparated (Table S4), the proposed CLE-CEC system without organic solvent in running buffer can realize better enantioseparation than other reported CLE-CEC systems (Table S5), paving a new way for chiral separation.

3.5. *Exploration of chiral recognition mechanism in CLE-CEC*

The chiral separation principle of CLE is based on the exchange between enantiomers and the ligands in the binary complex which is formed with the central metal ion. Then the enantiomers, ligand and central metal ion can form ternary complexes [15]. The chiral separation can be realized by the different stabilities of the ternary complexes as shown in the equations:

$$M(\text{L-Ligand})_n + \text{l-AA} \rightarrow M(\text{l-AA})(\text{L-ligand})_{n-1} + \text{L-Ligand} \quad (1)$$

$$M(\text{L-Ligand})_n + \text{d-AA} \rightarrow M(\text{d-AA})(\text{L-ligand})_{n-1} + \text{L-Ligand} \quad (2)$$

M is the central metal ion; L-Ligand is the chiral ligand; D-AA and L-AA is the amino acid enantiomers.

The three tested analytes (Dns-D,L-Gln, Dns-D,L-Met and Dns-D,L-Ser) could be baseline separated under the optimized conditions with the proposed CLE-CEC system. However, when the bare capillary was used, Dns-D,L-Gln could not obtain baseline separation even with the Zn(II)-L-Arg complexes in running buffer as the chiral selector (Table S4). This result indicated that the differences in stability of the formed ternary complexes were quite limited and the favorable enantioseparation could not achieve. While, when P(MMA) moiety of the block copolymer was used as the immobilized ligand and there is no free L-Arg in running buffer, none of the three pairs of Dns-D,L-AAs could be baseline separated, exhibiting that the chiral recognition ability of the immobilized ligands was also not satisfied. It should be mentioned that only syner-

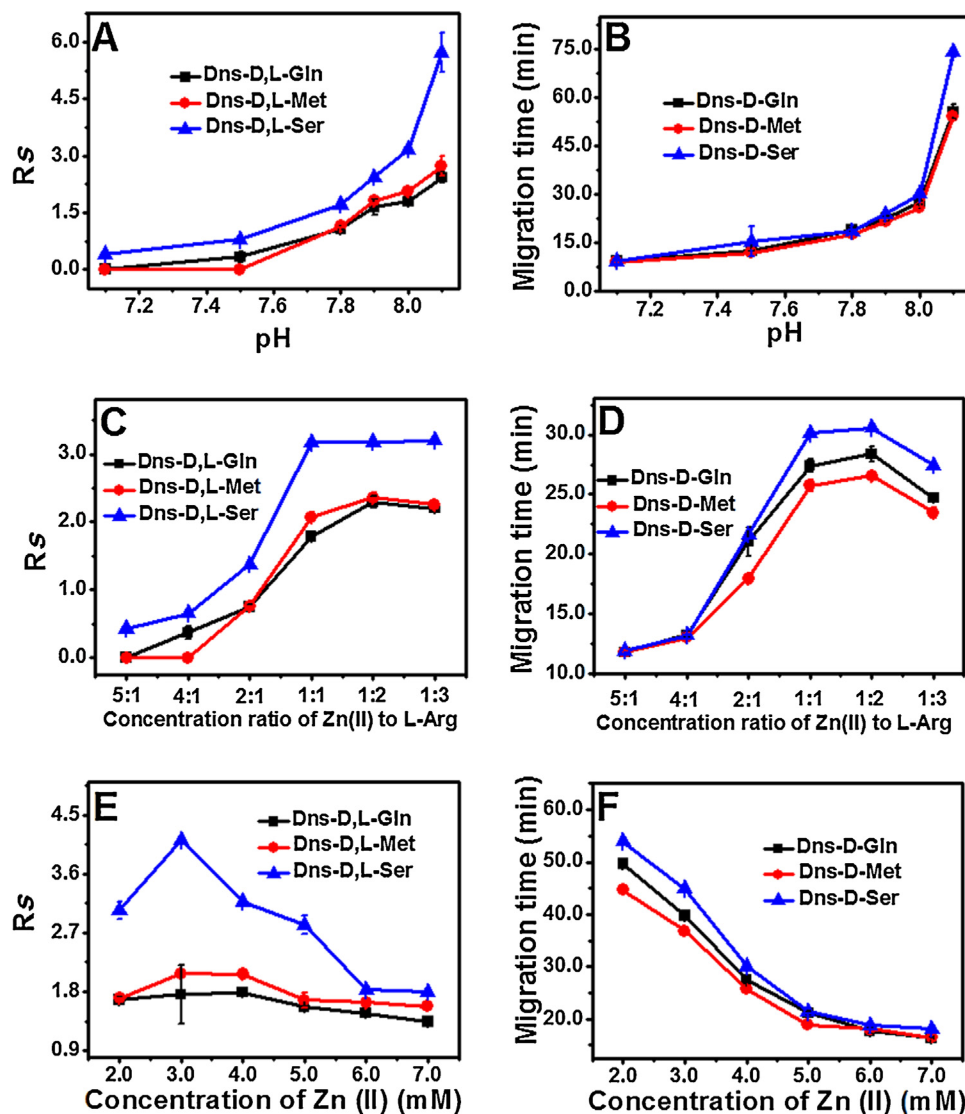

Fig. 3. Effect of buffer pH on enantioresolution *Rs* (A) and migration time (B). CLE-CEC conditions: 100.0 mM boric acid, 10.0 mM ammonium acetate, 4.0 mM Zn(II), 4.0 mM L-Arg at different pH from 7.1 to 8.1. Effect of the concentration ratio of Zn(II) to L-Arg on enantioresolution *Rs* (C) and migration time (D). CLE-CEC conditions: different concentration ratios of Zn(II) to L-Arg from 5:1 to 1:3, pH 8.0; other conditions are the same as A, B. Effect of concentration of Zn(II) on enantioresolution *Rs* (E) and migration time (F). CLE-CEC conditions: different concentrations of Zn(II) from 2.0 mM to 7.0 mM, the concentration ratio of Zn(II) to L-Arg was 1:1; other conditions were same as A, B. Applied voltage: −20 kV; injections, siphoned for 8 s in 15 cm height; detection wavelength, 254 nm; polymer 2 coated capillary, 75 μm i.d. × 60 cm (45 cm effective).

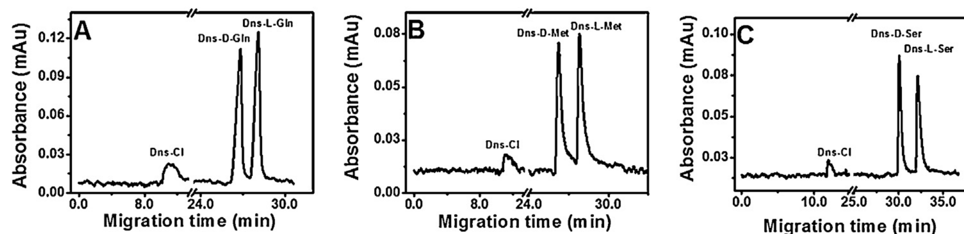

Fig. 4. Enantioseparation of three pairs of Dns-D,L-AAs under the optimum conditions. CLE-CEC conditions: 100.0 mM boric acid, 10.0 mM ammonium acetate, 4.0 mM Zn(II), 4.0 mM L-Arg at pH 8.0. Applied voltage: −20 kV; injections, siphoned for 8 s in 15 cm height; detection wavelength, 254 nm; polymer 2 coated capillary, 75 μm i.d. × 60 cm (45 cm effective). Dns-D,L-Gln (4.6 mM) (A), Dns-D,L-Met (4.5 mM) (B) and Dns-D,L-Ser (6.3 mM) (C).

gistic ligands, the immobilized PMAA ligand in block copolymer and free L-Arg ligand in buffer solution, can induce much enhanced enantioresolution in the proposed CLE-CEC system, as displayed in Table S4. Therefore, the chiral recognition mechanism of the developed CLE-CEC system was presumed to be the coordinating ligands effect, as exhibited in Fig. 5.

3.6. Quantitative analysis of D,L-Gln

The quantitative analysis of Dns-D,L-Gln was developed by the new constructed CLE-CEC system. The linear relationship based on the corrected peak area [31] and the Dns-D-Gln or Dns-L-Gln concentrations were shown in Table S6. The results proved that

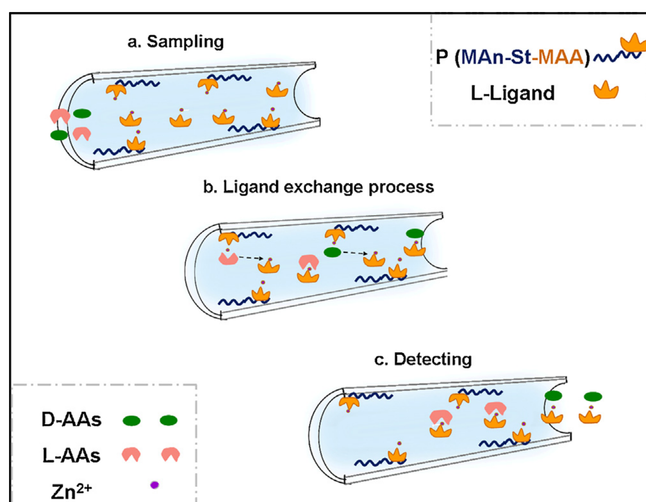

Fig. 5. Mechanism illustration of CLE-CEC separation process.

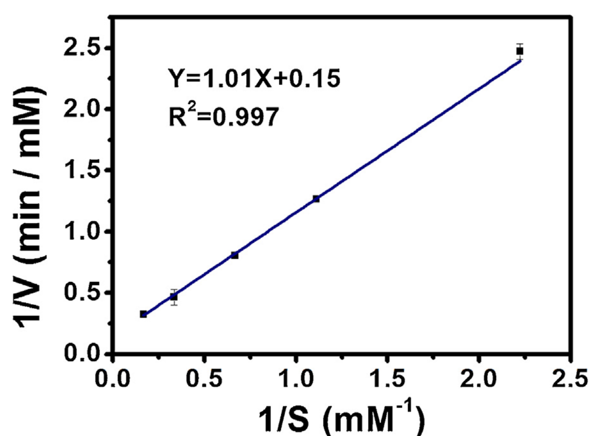

Fig. 6. Lineweaver–Burk plots of free glutaminase.

the CLE-CEC system could be expected for glutaminase enzymatic kinetics study with L-Gln as the substrate.

3.7. Kinetic study of glutaminase

It has been reported that glutaminase is a kind of hydrolytic enzyme, which could catalyze the conversion of L-Gln to L-Glu. The enzyme has been popularly applied in clinical and metabolites analysis [9]. In order to confirm the accuracy of the proposed CLE-CEC method, the kinetic study of glutaminase was investigated. L-Gln was used as the substrate and the hydrolysis efficiency was calculated through change of the L-Gln peak area measured by the established CLE-CEC assay.

The kinetic constants of enzyme reaction K_m and V_{max} were calculated by Michaelis-Menten equation [15]:

$$[S]/V = K_m/V_{max} + [S]/V_{max} \quad (3)$$

Where [S] is the concentration of the substrate, V is the enzyme reaction rate. K_m is Michaelis constant, which represents the affinity of the enzyme, and V_{max} reflects the enzyme activity and means the maximum reaction velocity. K_m and V_{max} of the free enzyme solution were calculated as 6.7 ± 0.85 mM and 6.7 ± 0.75 mM/min, respectively (Fig. 6). Three replicates (n = 3) were measured for each concentration value. The data was similar as that reported in the reference [32], demonstrating the feasibility of CLE-CEC protocol in practical application.

4. Conclusion

In this work, a novel CLE-CEC system was constructed for the first time with block copolymer P(MAn-St-MAA) as the coating and without organic solvent in running buffer. Comparing with the bare capillary, the enantioresolution of Dns-D,L-AA could be greatly enhanced on the coating one based on the synergistic ligands effect of the immobilized PMAA in coating and free L-Arg in buffer solution. The coated capillary exhibited good stability and repeatability. The results displayed that well enantioseparation of Dns-D,L-AAs could be obtained by the proposed CLE-CEC protocol, indicating its favorable power in the chiral analysis. Furthermore, the developed CLE-CEC system was successfully applied in the kinetics study of glutaminase with L-Gln as the substrate, showing the great potential of CLE-CEC method in bio-analysis.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.03.031.

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