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PAPER

Monolith and coating enzymatic microreactors of L-asparaginase: kinetics study by MCE–LIF for potential application in acute lymphoblastic leukemia (ALL) treatment†

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The study of enzyme immobilization using an extracorporeal shunt system is essential to eliminate the side effects of L-asparaginase (L-Asnase; including hepatic toxicity, allergic reaction, pancreatitis, central nervous system toxicity and decreased synthesis of blood clotting factors) when it was applied as an anticancer drug given directly to patients by injection. Thus, the novel monolith and coating enzymatic reactors of L-asparaginase were provided in this assay and a microchip electrophoresis–laser induced fluorescence (MCE–LIF) method was set up for the enzyme kinetics study. The enzymatic reactors would be a promising *in vitro* therapeutic method in an extracorporeal shunt system for acute lymphoblastic leukemia (ALL) treatment. For the first time, L-asparaginase was covalently bound to the polymer monolith and coating in the capillary and the activity characteristics of these enzymatic microreactors have been probed by Michaelis–Menten kinetic constants. Meanwhile, the D,L-amino acids were chirally separated using microchip electrophoresis with a laser induced detector and D,L-aspartic acid (D,L-Asp) were tested for the L-asparaginase enzymatic reactor kinetics study. Furthermore, human serum adding with L-asparagine (L-Asn) as the sample was hydrolyzed by the enzymatic microreactors. The results demonstrated that the developed enzymatic microreactor of L-asparaginase would be a potential therapeutic protocol for ALL treatment.

Introduction

Acute lymphoblastic leukemia (ALL) is a type of cancer of the white blood cells that is characterized by excess lymphoblasts. Since 1967, L-asparaginase (L-Asnase) has been used as an integral part of the treatment of child ALL.[1] It can catalyze the hydrolysis of L-asparagine (L-Asn) to L-aspartic acid (L-Asp) and ammonia. Thus, the relative deficiency of L-Asn in the serum and endogenous L-Asn synthesis could cause the apoptosis and impair protein synthesis in leukemic blasts.[2] Results showed that L-Asnase could cause complete remission in 40–60% of paediatric ALL cases when it was used alone.[1] Although L-Asnase is effective in the treatment of malignant diseases, many side effects, including hepatic toxicity, allergic reaction, pancreatitis, central nervous system toxicity and decreased synthesis of blood clotting factors, have been reported about this standard chemotherapy component for ALL.[3] It was well documented that L-Asnase was the leading cause of hypersensitivity reactions in 5 to 45% of the patients[4] and thrombotic events varying from 1.1 to 73% in children.[5,6] In addition, because the L-Asnase was a bacterial protein and might be attributed to antibody formation when given to human beings, it could lead to not only allergic reactions but also influence the enzyme's activity and be dose limiting.[7] These disadvantages against L-Asnase will limit the use of the drug and terminate some patients' therapeutic plan.

To avoid the side effects of L-Asnase mentioned above, such as hepatic toxicity and allergic reaction when it was injected to patients, and to take the most advantage of an enzyme treatment method, L-Asnase can be chemically modified,[3] entrapped or immobilized into other carriers.[8] Among these methods, the extracorporeal shunt system (ESS) with an immobilized enzymatic reactor provides an entirely new idea for the treatment of leukemia and deserves to receive special attention. ESS methods were based upon embedding L-Asnase in columns or immobilization on the tube or hemodialysis film or outside of the hollow fiber hemodialyzer,[9] then the blood flowed through the enzymatic reactors to reduce the concentration of plasma L-Asn significantly, thereby depriving tumor cells of this nutritional amino acid.[10,11] The method operated without the injection of L-Asnase into patients and the enzyme did not directly contact with the organs, thus the allergic reaction and toxicity can be avoided effectively. Moreover, the clinical application of L-Asnase in ESS

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has advantages over the injection of large amounts of enzyme, precluding the observed toxic and untoward immunological effects.

However, several limitations have been relatively explored, including loss of activity during coupling the enzyme to the reactor, low surface area, and potential alteration of enzyme structure and function.[12,13] Since the discovery of the advantageous property of immobilized enzymes, attempts have been made to explore new substrate materials with appropriate structures (including the morphology and surface functionality) and compositions to improve the catalytic efficiency of the immobilized enzymes.[14–16] Among these materials, monolithic column enzymatic reactors profit from the merits of less toxicity and large surface area, have higher enzyme concentrations that lead to shorter digestion time and reduce chemical denaturants of the enzyme, and have provided a suitable material for enzyme immobilization.[17] Thus, following our success with the glycidyl methacrylate-*co*-ethylene glycol dimethacrylate (GMA-*co*-EDMA) monolithic column as a separation medium[18] and immobilization of proteins for chiral separation,[19] the use of the GMA-*co*-EDMA monolith and coating enzymatic reactors for immobilization of L-Asnase could be well established. We had anticipated that the application of the GMA-*co*-EDMA monolith would be well-suited for the fabrication of an enzymatic microreactor for the treatment of ALL.

In order to understand further the activity of L-Asnase enzymatic reactors, a microchip electrophoresis–laser induced fluorescence (MCE–LIF) method has been developed for the detection of L-Asn in previous studies.[20,21] This method has substantial advantages over conventional analytical technologies (such as spectrophotometric assays and chromatography methods[22]) including high speed, low consumption of sample and buffer, miniaturization and non-toxicity. Because amino acid enantiomers in biomedical samples have played different roles related to disease therapy and might interfere in the separation and quantitative analysis of the substrate and the product of L-Asnase, therefore, it is meaningful to develop an MCE method for the simultaneous chiral separation and determination of amino acids.

Herein, we attempt to develop an ESS therapeutic protocol of ALL by using monolith and coating (GMA-*co*-EDMA) enzymatic reactors of L-Asnase. The enzyme activities of the enzymatic reactors were observed by the MCE–LIF method. Meanwhile, D,L-amino acids were chirally separated by introducing γ-CD into the running buffer as the chiral selector. Furthermore, the ESS method of the L-Asnase enzymatic reactor described here is also applicable to L-Asn in human serum samples to assess the potential for use in real physiological and clinical research.

Materials and methods

Materials

Amino acid samples and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical (St. Louis, MO, USA). L-Asnase was purchased form ProSpec-Tany Techno-Gene (Rehovot, Israel) and it was produced from *Escherichia coli* containing 303 amino acids and had a molecular mass of 31 731 Da.

Ethylene dimethacrylate (98%, EDMA) and glycidyl methacrylate (97%, GMA) were purchased from Acros (New Jersey, USA). 3-(Trimethoxysilyl)propyl methacrylate (98%, γ-MAPS) was obtained from Sigma (USA). 2,2′-Azobisisobutyronitrile (AIBN) was produced by Shanghai Chemical Plant (Shanghai, China) and refined before use. Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), cyclohexanol and dodecanol were from Fuchen Chemical Plant (Tianjin, China). Benzamidine was obtained from Amresco. Other reagents were all analytical grade.

All aqueous solutions were prepared with triple distilled water produced by the distillation apparatus Yarong Biochemical Instrument model SZ-97 (Shanghai, China), then filtered through a 0.45 μm membrane before use and stored at 4 °C. The electrophoretic buffer was 25 mM borate solution (pH 9.8) containing 30 mM SDS and 12 mM γ-CD. The 2.0 mM FITC solution was prepared in acetone. Stock solutions of 2.0 mg mL^{-1} D,L-amino acids were prepared in 25 mM borate buffer (pH 9.8) and derivatization with FITC as in the method of ref. 20. The L-Asnase (4.5 units mL^{-1}) was prepared in 50 mM Tris-HCl buffer (pH 8.7) and stored at −18 °C. Working solutions were diluted from the stock solutions with water by 10–10^4-fold.

MCE–LIF

Separation experiments of amino acids were carried out on an intelligent six-path-high-voltage electric device MCE–LIF analyzer (Jinan, China).[23,24] The analyzer had a confocal mode detection system with a 473 nm semiconductor double-pumped solid-state laser (20 mW) and an emitted fluorescence, filtered spectrally (520 + 10 nm) by a narrow band filter and detected by a photomultiplier tube.

The chiral separations of amino acids were carried out using a 'cross channel' glass chip provided by Dalian University of Technology. The microfluidic channel was rinsed sequentially with 0.1 M NaOH, water, and electrophoretic buffer solution for 10 min each before runs started. The buffer reservoir (B) and sample waste reservoir (SW) were filled with buffer, then a vacuum was used at the buffer waste reservoir (BW) in order to fill the separation channel with buffer and 10 μL sample solution was added in sample reservoir (S). The voltages for the injection and separation are shown in Table 1.

Coating and monolith enzymatic reactors

The monolithic enzymatic reactors were made using the GMA-*co*-EDMA monolith as reported before.[18] Firstly, the outer coating of fused-silica capillaries (75 μm I.D.) was removed by

Table 1 Typical output voltage program for injection and separation

Time section		Injection	Separation
Run time/s		40	400
Applied voltage/V	B	300	2500
	BW	450	0
	S	900	1600
	SW	0	1600

a sharp shaver to make them UV transparent and the internal wall was vinylized to enable covalent attachment of the monolith to the walls.[25] Briefly, the walls of the capillaries were activated with a 1.0 M NaOH solution for 2 h, washed with water followed by 1.0 M HCl for 5 min, rinsed with water and acetone, and dried with a stream of nitrogen. The capillary was then rinsed with a 50% solution of γ-MAPS in THF and allowed to equilibrate for 12 h at room temperature. According to the method reported previously,[26,27] a vacuum pump (Yarong Biochemical Instrument, Shanghai, China) was applied to obtain a negative-pressure (0.09 MPa) in a bottle that can let the polymerization mixture solution pass through the capillary uniformly and reproducibly. Then a poly(GMA-*co*-EDMA) polymerization mixture (0.50 mL GMA, 0.50 mL EDMA, 0.25 mL dodecanol, 1.75 mL cyclohexanol and 30 mg AIBN) was draw through the capillary for 5 min and sealed after the mixture was homogenized in an ultrasonic bath for 10 min and purged with nitrogen for 5 min. After the polymerization was initiated by placing the capillaries under a UV lamp at 254 nm and irradiating for 30 min, the column was rinsed with methanol to remove the porogen in the monolith.

The coating was fabricated using the same method as with monolith enzymatic reactors except that the polymerization mixture was blown off by nitrogen to get an open tubular monolithic coating in the capillary.

The characterization of the coating and monolith enzymatic reactors were detected using an S-4300 scanning electron microscope (SEM, Hitachi, Japan). The covalent immobilization of enzyme was using a syringe pump (Lange, Hebei, China).

Immobilization of L-Asnase

The immobilization process was carried out at room temperature. Before immobilization, the coating and monolithic enzyme supports were firstly equilibrated for 30 min with 50 mM Tris-HCl buffer (pH 8.7). The L-Asnase (4.5 units mL^{-1}) was dissolved in Tris-HCl buffer (pH 8.7) and 50 mM benzamidine was added to avoid undesired autodigestion. At first, the L-Asnase solution was rinsed through the coating and monolith enzymatic reactors for 1–10 h, then the enzymatic reactors conjugated with L-Asnase were flushed with 50 mM Tris-HCl buffer including 0.5 M NaCl to eliminate non-specific physically adsorbed L-Asnase. The enzymatic reactors should be stored in 50 mM Tris-HCl buffer (pH 7.6) containing 10 mM $CaCl_2$ and 0.02% NaN_3 at 4 °C when left unused.

The amounts of L-Asnase bound to the coating and the monolithic enzyme supports were measured using a Bradford assay as mentioned in refs. 28 and 29. Briefly, the enzymatic reactors were firstly immobilized by L-Asnase for 8 h in 8.0 cm capillaries, and then the concentrations of the L-Asnase solution were determined before and after passing though the coating and the monolithic enzyme supports. Each of the standard solutions (1–100 μg mL^{-1}) as well as the enzyme solution sample was placed in vials and incubated with Bradford reagent for 5 min. Finally, the absorbance of each solution was measured using a spectrophotometer at 595 nm, and the enzyme concentrations were calculated.

The activities of immobilized L-Asnase were evaluated by pumping 50 μL of L-Asn solutions (15–530 μM) in a 50 mM Tris buffer (pH 8.0) using a syringe pump through the monolith at a flow rate of 100 μL h^{-1} with detection of the resulting products of L-Asp by MCE–LIF.[30] The effect of substrate concentration on the initial rate of the reaction was investigated by varying the concentration of L-Asn and the Michaelis constant (K_m) and maximum velocity (V_{max}) values were calculated from the data using the Lineweaver–Burk method.

Biological sample preparation

According to refs. 9–11, human serum added with L-Asn was used to evaluate the performance of the enzyme reactors. Human serum samples were obtained from the Medical Experiment and Analysis Center, PLA General Hospital (Beijing, China). The serum samples, in 5.0 mL vials, were put into boiling water for 15 min and were centrifuged for 10 min (10 000 rpm) to eliminate the proteins. The D,L-Asn were added into the supernatants (1 : 1, v/v) and flowed though the coating and monolith enzymatic reactors for 30 min at a flow rate of 100 μL h^{-1}. Then the collected solution was derivatized with FITC and diluted for future analysis.[20] Experimental protocols involving the use of human blood were reviewed and approved by the Institutional Review Board of Medical Experiment and Analysis Center, PLA General Hospital, Beijing, China.

Results and discussion

In this study, we demonstrate a kind of polymer monolith enzymatic reactor in an ESS for the treatment of ALL. Since the activities of the coating and the monolith enzymatic reactors should be characterized by the values of the K_m and V_{max}, thus it is essential to establish a method for the separation and the quantification analysis of the product (L-Asp).

Separation of amino acid enantiomers

Recently, we have succeeded in amino acids separation and an L-Asnase kinetics study using an MCE method with LIF detection.[20] Herein, MCE–LIF was presented also for the enantiomeric separation of amino acids after precolumn derivatization with FITC. A cross-channel glass microfluidic chip with a 7.0 cm long separation channel was used for chiral separation and highly sensitive detection was obtained by LIF detection. The γ-CD, which has often been used as a facile, robust and highly efficient chiral selector in amino acids chiral separation, has been chosen for the chiral selection in the running buffer applied in MCE. The optimized experimental conditions for the chiral separation of amino acids were 25 mM borate buffer containing 12 mM γ-CD and 30 mM SDS at pH 9.8, which are the same as mentioned in ref. 31.

Carrying out the chiral separation of amino acid enantiomers, especially for D,L-Asn and D,L-Asp, in complex biological matrices and the enzyme kinetics study of L-Asnase in enzymatic reactors by MCE has been a challenge due to the short separation channel on the microchip compared to capillary electrophoresis. Because other kinds of amino acids in biological samples might interfere with the quantification analysis of D,L-Asn and D,L-Asp, thus, 14 kinds of amino acid enantiomers were tested and the results have been shown in Table 2. The results showed that seven kinds of amino acids gave chiral resolution

Table 2 Enantiomer separation of amino acids

	Migration time		
Amino acids	t_D/min	t_L/min	R
Ala	2.29	2.38	1.74
Glu	3.03	3.19	1.61
Asp	3.88	4.05	1.61
Asn	2.55	2.63	1.38
His	2.36	2.45	1.36
Tyr	2.32	2.38	1.23
Try	2.22	2.29	1.08
Leu	2.00	2.09	0.98
Met	1.75	1.80	0.95
Ser	2.35	2.41	0.86
Phe	2.14	2.18	0.82
Lys	1.81	1.83	0.63
Thr	2.41	2.45	0.42
Val	2.34	2.35	0.19

values (R) from 1.0 to 1.7 and R values for the other seven amino acids were below 1.0. According to the migration time, although D,L-Asn would most likely be interfered with by other amino acids, for D,L-Asp, which migrated at last, this would not suffer from any interference by other amino acids in its determination. Moreover, using this method, enantiomers of D,L-Asp could be well separated (resolution: 1.61). A typical electropherogram of D,L-Asn and D,L-Asp is shown later in Fig. 4A.

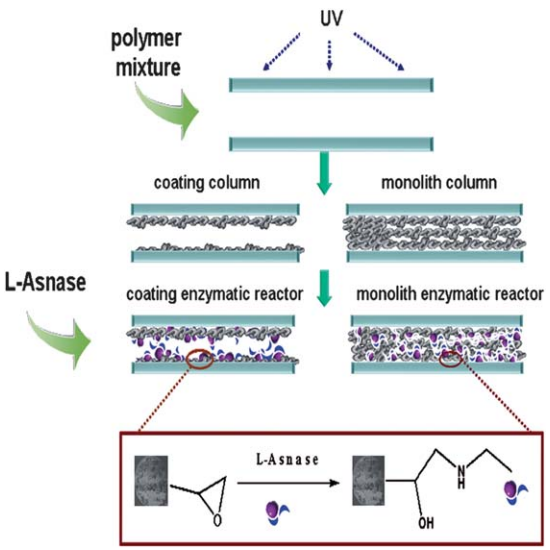
Owing to be the product of L-Asnase and non-interference with other amino acids and substances in the human serum sample, L-Asp was set as the target for the following determination. The present MCE–LIF chiral separation method for D,L-Asp was evaluated in terms of the response linearity, limot of detection (LOD) and reproducibility. Six-point calibration curves were detected by analyzing a series of D,L-Asp standard solutions at different concentrations and the peak heights were used for quantification. The results are summarized in Table 3. The reproducibilities of the analytes response (RSD %) in terms of both peak height and migration time were tested by separating a standard solution for five times and the RSDs for all analytes were found to be 2.1 and 4.0%, respectively. These results displayed that the method was suitable for the quantification analysis of D,L-Asp.

Preparation and the enzyme activities of the enzymatic microreactors

The specificity of L-Asnase and its ability to catalyze L-Asn hydrolyzation reactions have made it attractive for applications in the treatment of ALL. Nevertheless, free enzymes usually have low operational stability, and the preserving of the free enzyme is not feasible in general. These drawbacks have somehow hindered their application in treatment processes. Coating and monolith enzymatic reactors may be good strategies for enzyme immobilization. Poly(GMA-*co*-EDMA) monolithic polymerization mixtures were selected owing to the merits of its epoxy radical which could conveniently react with an enzyme. The polymerization mixtures used for the preparation of monolithic materials *in situ* were inspired from those we used before.[18] However, considering that the temperature has a significant effect on the porous properties of the monolith is much time consuming, the UV-initiated polymerization at room temperature was chosen in this study. Brief introduction to the polymerization and the immobilization of L-Asnase are shown in Scheme 1.

In this work, we report two methods (coating and monolithic porous materials) for the preparation of reactive poly(GMA-*co*-EDMA) porous microreactors, their use as supports for the immobilization of L-Asnase, and the application as an ESS treatment for ALL. Scheme 2 illustrates the potential application of the enzymatic microreactors for ESS in ALL treatment.

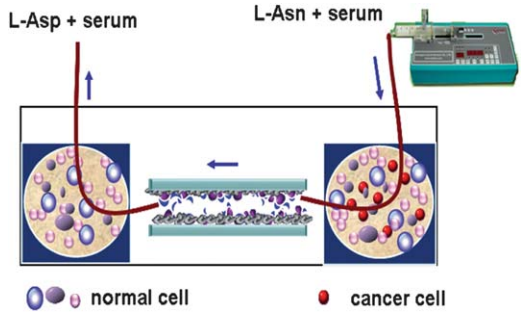
The SEM images of the coating and the monolithic porous materials have been illustrated in Fig. 1. The internal morphologies of monolithic (Fig. 1A,B) and coating (Fig. 1C,D) enzymatic reactors demonstrate that the porous polymers are well-distributed and homogeneous across the entire capillaries. Moreover, it has been found that most macropores had a diameter of around 1–2 μm for the monolith. These images also reveal that open-tubular enzymatic reactors have a thick coating with a kind of granular stack structure clearly expanded to form



Scheme 1 Brief introduction to the polymerization of enzymatic reactors and the immobilization of L-Asnase.

Table 3 Linear ranges and detection limits of D,L-Asp

Analytes	D-Asp	L-Asp
Linear regression equation	$y = 4.6 \times 10^2x + 2.6 \times 10^3$	$y = 4.3 \times 10^2x + 2.3 \times 10^3$
Linear ranges/μM	$5.0–6.0 \times 10^2$	$5.0–6.0 \times 10^2$
R^2	0.993	0.994
LOD/μM	1.5	1.5



Scheme 2 Diagram of ESS using the enzymatic microreactors.

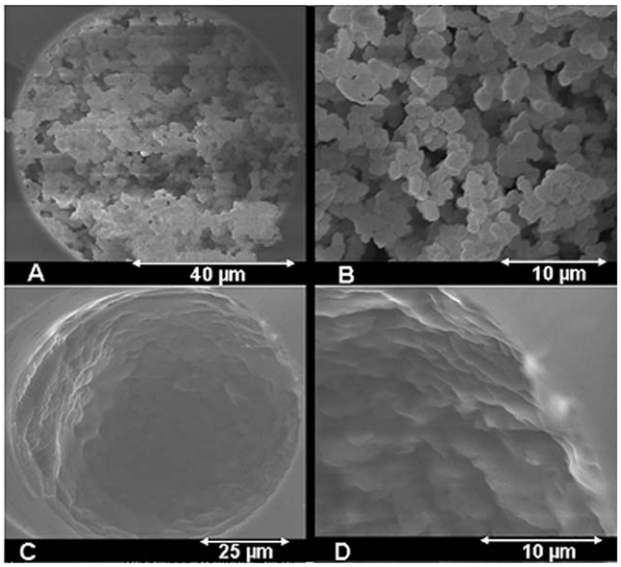


Fig. 1 SEM of monolith and coating in capillary: (A,B) monolith, (C,D) coating.

a rough surface. Interestingly, the unique properties make these supports superior as enzymatic reactors.

In order to evaluate the amounts of the enzyme immobilized on the optimized coating and the monolithic supports, the concentrations of the enzyme solutions before and after the immobilization were determined. Enzyme concentration decreases of 8.3 μg mL^{-1} and 14.4 μg mL^{-1} were found for the coating and the monolithic supports, respectively, in the enzyme immobilization procedures. The results were comparable to the enzymatic reactors reported in ref. 29.

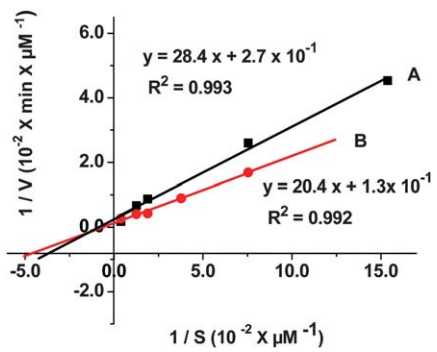


Fig. 2 Lineweaver–Burk plots for L-Asnase immobilized on monolith (A, ■) and coating (B, ●) enzymatic microreactors.

To investigate the enzyme activities of the microreactors, K_m and V_{max} were investigated for the coating and monolith enzymatic reactors. Fig. 2 illustrates the Lineweaver–Burk plot of the two enzymatic reactors and K_m and V_{max} values were derived from the linear form of the Michaelis–Menten equation. The kinetics data were $K_m = 3.8$ μM, $V_{max} = 106.2$ μM min^{-1} for monolith and $K_m = 7.7$ μM, $V_{max} = 157.4$ μM min^{-1} for coating enzymatic reactors, respectively. These results were similar with the data in the ref. 10. These results enable an evaluation of different supports of enzyme effects. Compared to free enzyme reaction,[20] immobilization L-Asnase has lower K_m value which means that a higher reaction rate can be obtained at a lower concentration of L-Asn in the substrate solution.

The microreactor prepared from coating support exhibits a higher maximal velocity than the reactor based on the monolith, while the Michaelis constants are almost equal for both, indicating that the former has a high activity for hydrolyzation. On the other hand, the coating enzymatic microreactor has a low flow pressure and is convenient for the application of the ESS, so the coating microreactor was used for enzyme immobilization in the following experiments.

In order to optimize the immobilization condition of the enzymatic reactors, the time of enzyme bimolecular reaction in solution with the surface of the solid support and the length of the capillaries, which could influence the microreactors catalytic activation, have been investigated. The results have been displayed in Table 4 and in Fig. 3. An increase in reaction time and in the length of the capillaries should make the microreactors to get higher loading of the active enzyme since the immobilization is a bimolecular reaction between L-Asnase and the epoxy functionalities at the surface of the solid support. As displayed in Table 4 and in Fig. 3, it could be found that the increase in the reaction time of L-Asnase used for immobilization from 1 to 10 h affects the yield of the products and the activity of the microreactors. Similarly, the increased length of the enzymatic reactors also leads to an increase in activity. However, the prolonged immobilization time and increased length of the enzymatic reactors would be at the risk of the loss stability of the enzyme in the immobilization process and the high pressure of the enzymatic reactors. Thus, an 8 cm length enzymatic reactor with immobilization for 8 h was chosen finally.

For testing the operational stability of the immobilized enzyme, the amount of L-Asp at the reactor outlet was determined periodically (7-day intervals) using the MCE–LIF method.[32] After 21 days of continuous observation, the decrease in L-Asp values was not detected (RSD = 3.7%), but it decreased to be a half at 28 days.

Simulation of perfusion studies

Previous studies[3,9] have shown that the concentration of L-Asn in normal serum ranges from approximately 30 to 60 μM in untreated human cancer patients. Moreover, Horvath *et al.*[10] have used L-Asn added to the perfusate to obtain a 100 μM substrate concentration. So in this study, 135 μM L-Asn was added in the normal human serum and the serum samples were

Table 4 Effect of different immobilization time on the activities of the coating enzymatic reactors

Enzymatic reactor	Immobilization time/h	Concentration of L-Asp/μM
1	1	27.0
2	4	41.9
3	8	71.1
4	10	90.5

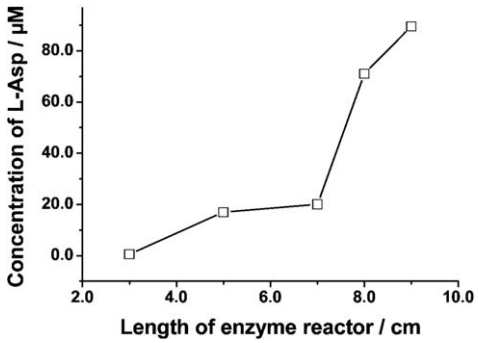


Fig. 3 Effect of capillary length on the activity of immobilized L-Asnase.

flowed into the enzymatic reactors for 30 min. The results are illustrated in Fig. 4. The L-Asp concentration in the serum rapidly increased due to the reaction of the enzymatic reactor on the L-Asn. Moreover, it has been found that the whole blood samples without treatment mixing with D,L-Asn could also be hydrolyzed by the capillary enzymatic reactors (Electronic Supplementary Information, Fig. S1†).

These experiments clearly showed that by perfusing the normal human serum sample and the whole blood sample with L-Asn into the enzymatic reactors, the L-Asn could be hydrolyzed mostly to become L-Asp. Thus, using the MCE–LIF method could allow the determination of L-Asp, which is the product of the enzyme reaction, without interference from other amino acids and substances in the serum and the whole blood samples. Furthermore, the enzymatic reactors made by the polymer materials could be used as the potential protocols employed in physiological studies of ALL treatment.

Conclusions

We optimized the conditions for immobilization of L-Asnase *via* poly(GMA-*co*-EDMA) materials and obtained the higher specific activity and specific productivity using polymer material coating enzymatic reactors. The enzymatic reactors therefore showed high operational stability and potential for continuous use in the ESS treatment for ALL. A sensitive and selective MCE–LIF method for the chiral separation of amino acids and kinetic studies of enzymatic reactors has been set up and optimized. Owing to the high specific activity of the immobilized L-Asnase compared with enzymes in free solution, L-Asn in the human serum samples was hydrolyzed efficiently and the product of the enzyme reaction, L-Asp, can be detected using the MCE method. These results indicate that this method of enzyme immobilization is a superior technique for L-Asnase and may be applicable for the ESS treatment of ALL.

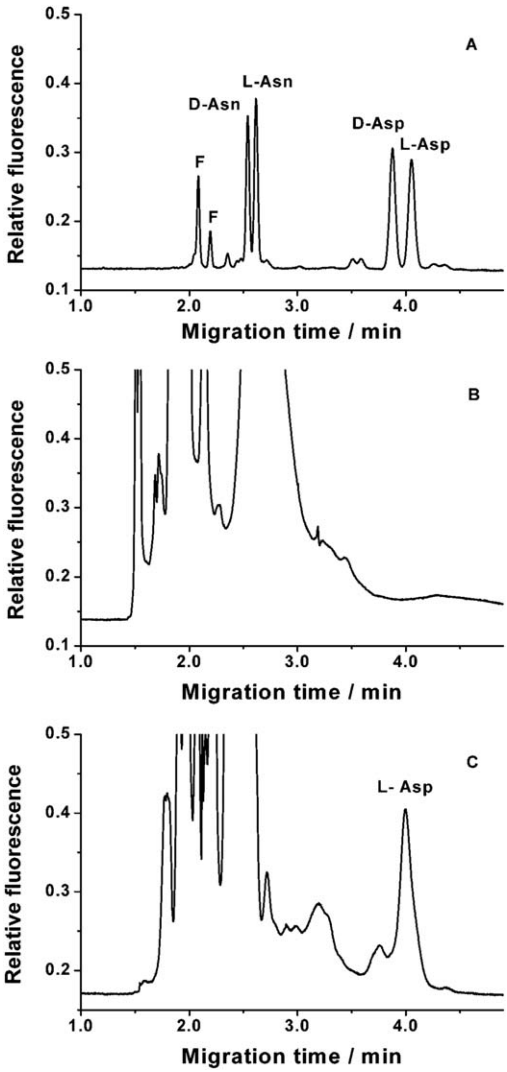


Fig. 4 Electropherograms of hydrolyzation of L-Asn in human serum samples using coating enzymatic reactor as an ESS. (A) standard of D,L-Asn and D,L-Asp; (B) human serum sample; (C) L-Asn added in human serum sample after being hydrolyzed using the coating enzymatic reactor.

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