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A Fine Match Between the Stereoselective Ligands and Membrane Pore Size for Enhanced Chiral Separation

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A D,L-tryptophan separation factor of 12–15 and D-tryptophan yield of >95% have been successfully achieved through using human serum albumin (HSA) as the stereoselective ligand in an affinity ultrafiltration (UF) system. The obtained separation factor in this work is even higher than the intrinsic value of 8.5 of HSA. This synergism may arise from the fact that a fine match between the regular crystalline structure of HSA molecules and suitable pore size of membranes makes some HSA molecules be retained within the membrane cross-section, thus offering a second-stage binding opportunity for L-tryptophan molecules. Therefore, a simultaneous enhancement in separation factor and D-tryptophan yield has been fulfilled in this work. The feasibility of HSA regeneration after D,L-tryptophan separation has also been demonstrated through a series of pH adjustment experiments. This study reveals the applicability of HSA in affinity UF systems for chiral separation due to economization of material costs. © 2009 American Institute of Chemical Engineers *AIChE J,* 55: 2284–2291, 2009
Keywords: chiral separation, affinity ultrafiltration, stereoselective ligand, membrane pore size, serum albumin

Introduction

Many pharmaceuticals and agricultural chemicals exist as racemic mixtures with similar physical and chemical properties. Usually, only one of the two enantiomers has the desirable biological effects and on the contrary, the other may exhibit toxic side effects. For example, L-tryptophan has been widely applied as an antidepressant agent; nevertheless, D-tryptophan has no biological effect.[1] Moreover, a higher excretion in human bodies often occurs when D,L-tryptophan or D-tryptophan is administered due to the higher metabolic degradation of D-tryptophan. As a result, the biological value of D,L-tryptophan mixture can only reach a half of neat L-tryptophan.[2,3]

Therefore, the importance of chiral molecules in pharmaceutical and food industries has generated a tremendous demand for the development of separation techniques in large-scale purification. At present, the most widely used

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technologies for separation of racemic mixtures include column chromatography,[4–6] salt crystallization,[7,8] counter-current-chromatography,[9,10] asymmetric catalysis,[11] and stereoselective enzyme catalysis.[12,13] However, these approaches have difficulty in scale-up operation, thus leading to a relatively low productivity, high energy consumption, and expensive consumables. Hence, membrane-based chiral separation techniques are attracting more and more attention because their various advantages make large-scale operation easier and more cost effectiveness.[14–19] Affinity ultrafiltration (UF) is one of membrane-based chiral separation techniques, wherein one of the two enantiomers is selectively bound by a large stereoselective ligand in a bulk solution and thus is held at the retentate side of UF membranes, whereas the other enantiomer relatively freely passes through UF membranes and concentrates at the permeate side of membranes.[20] The selection of stereoselective ligands is very important for affinity UF performance in chiral separation and usually needs to meet two criteria[21]: (1) they show a preference in binding one enantiomer to the other enantiomer in racemic mixtures; (2) their size is so large that they can be retained at the retentate side of UF membranes.

Albumins, an important protein family in blood, are perhaps one type of potentially promising stereoselective ligands in the affinity UF system because they have two prominent binding sites; namely, the warfarin-azapropazone site (site I) and the indol-benzodiazepine site (site II).[22–24] It is well known that L-tryptophan shows a highly selective binding to site II,[25] whereas D-tryptophan only exhibits an indirect interaction with site I.[26] The studies done by Higuchi et al.[17–19] have demonstrated the feasibility that albumin could be used for chiral separation in the affinity UF system. They achieved a separation factor as high as 7 for D,L-tryptophan racemic mixture when bovine serum albumin was used as the selective ligand.[17] Later, some researchers such as Poncet et al.[27] and Romero and Zydney[28,29] developed this system and raised the separation factor to 11 by optimizing the molar ratio of ligand to racemic mixture, buffer pH value, ionic strength, and so forth. Nevertheless, so far most researches mainly used bovine serum albumin (BSA) as a ligand and little work has been done on the application of other kinds of albumins in the affinity UF system.

Lagercrantz et al.[30] studied the stereoselective binding of tryptophan enantiomers to serum albumins from different species by affinity chromatography on columns of immobilized serum albumins. They found that the enantiomers of D,L-tryptophan have different binding constants to different kinds of serum albumins although L-tryptophan always showed a stronger binding capacity than D-tryptophan for all species investigated in their work. For BSA, the association constants of L-tryptophan and D-tryptophan are 1.5×10^{-4} M^{-1} and 0.23×10^{-4} M^{-1}, respectively, whereas for human serum albumin (HSA), the association constants of L-tryptophan and D-tryptophan are 1.1×10^{-4} M^{-1} and 0.13×10^{-4} M^{-1}, respectively. In the later study, Kosa et al.[31] compared the binding sites of HSA with those of bovine, canine, rabbit, and rat serum albumins, and attributed their functional difference to microenvironmental changes in the binding sites such as the size and hydrophobicity of the binding pocket. Therefore, the first objective of this work is to compare the performance difference between HSA and BSA as a stereoselective ligand in the affinity UF system for the separation of tryptophan racemic mixtures.

Up to the present, previous studies mainly used the UF membranes with a stipulated pore size for chiral separation, which could ensure a high rejection for the permeation of stereoselective ligand (i.e. BSA).[27–29] Little work has been done on the influence of membrane pore sizes on the separation factor and yield in the affinity UF system. Therefore, the second objective of this work is to optimize the separation factor and yield by intelligently matching the pore size of UF membranes with the three-dimensional structure of stereoselective ligand—HSA.

Experimental

Materials

The racemic mixture of D,L-tryptophan (99%) and neat L-tryptophan (99%) were obtained from Alfa Aesar (Pelham, NH). Bovine serum albumin (Sigma A-7906, >98%) and HSA (Sigma A-1653, 96-99%) were used as the stereoselective ligands. α-Cyclodextrine (α-CD) as a background electrolyte (BGE) was purchased from CycloLab (Budapest, Hungary). All hydrophilic polyethersulfone (PES) membranes used for filtration experiments were offered by NADIR, Wiesbaden, Germany. They had a nominal molecular weight cutoff (MWCO) of 5, 10, and 30 kDa, respectively, which were designated as Mem-5k, Mem-10k, and Mem-30k, respectively, in this work. Phosphoric acid (99%) and potassium dihydrogenphosphate (99%) used for phosphate buffer were purchased from Merck (Darmstadt, Germany) and Nacalai Tesque (Kyoto, Japan), respectively.

Affinity UF experimental procedure

The racemic solution with a concentration of 0.1 mM was first prepared by dissolving D,L-tryptophan in a 10 mM phosphate buffer with a pH value of 8.0. Second, a certain amount of stereoselective ligands (i.e. BSA or HSA) were added to the tryptophan solution under a vigorous stir to achieve a molar ratio of 2:1 of ligand to tryptophan. Third, after reaching a binding equilibrium via stirring for 3 h, the tryptophan–ligand mixture solution was fed into the self-designed dead-end UF cell with a diameter of 70 mm for membrane filtration experiments. Before chiral separation, all flat sheet PES membranes with a diameter of 60 mm were flushed with deionized water to remove glycerol, which was used to maintain the membrane pore structure. Finally, the permeate samples were collected periodically for offline determination of tryptophan concentrations. The temperature and pressure were always kept at room temperature and 2 bar, respectively, for all membrane filtration experiments.

Measurements of tryptophan concentration by capillary electrophoresis

Tryptophan concentrations were measured by capillary zone electrophoresis using a 50 mM α-CD phosphate solution as a BGE at pH = 2.2. 15 μl of 1 M HCl/0.2 M KCl solution was added into 1 ml collected permeate sample before injecting samples into capillary zone electrophoresis to prevent the overlap of D-tryptophan and L-tryptophan

peaks in electropherograms which often happens at medium and high pH values. Electrophoresis was carried out using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) equipped with a diode-array detector. The system was manipulated via a 32 Karat 8.0 software. Data were acquired using a 50-cm long-fused silica capillary (UN-DEACT Capillary Electrophoresis (CE) column with an inner diameter of 75 μm; Agilent Technologies, USA) under an applied voltage of 25 kV. The wavelength for detection was 214 nm, and the tryptophan concentration was determined by integrating the peak area in electropherograms.

The separation factor (α) and the yields of L-tryptophan and D-tryptophan (Y_L and Y_D) in the affinity UF system are defined as follows[17,28]:

$$\alpha = \frac{C_{p,D}/C_{f,D}}{C_{p,L}/C_{f,L}} \quad (1)$$

$$Y_L = \frac{C_{p,L}}{C_{f,L}} \times 100\% \quad (2)$$

$$Y_D = \frac{C_{p,D}}{C_{f,D}} \times 100\% \quad (3)$$

where $C_{p,D}$ and $C_{p,L}$ denote the concentrations of D-enantiomer and L-enantiomer at the permeate side of membranes, respectively, while $C_{f,D}$ and $C_{f,L}$ express the concentrations of D-enantiomer and L-enantiomer at the feed side of membranes, respectively.

Membrane characterization

The distribution profile of nitrogen element inside the membranes was measured by the Oxford (Edinburgh, UK) energy dispersion of X-ray (EDX) to trace the distribution of HSA molecules after passing through the membranes with different pore sizes. Membrane samples were prepared by fracturing in liquid nitrogen. After mounting all the specimens on the stub by using double-sided conductive carbon adhesive tape, they were sputter coated with platinum using JEOL JFC-1200 ion sputtering device before testing. All EDX tests were performed using a line scan mode for 90 s along the cross-section of PES membranes.

HSA regeneration

An experiment was designed by pH adjustment to prove that HSA as a stereoselective ligand could be regenerated for tryptophan separation. First, a 0.1 mM neat L-tryptophan solution was prepared at pH = 7.4 and tested by CE. Second, a stipulated amount of HSA was added to the L-tryptophan solution at pH = 7.4 to reach a molar ratio of 2:1 of HSA to L-tryptophan for the CE analysis at the second time. Third, a 1 M HCl/0.2 M KCl solution was added to the L-tryptophan-HSA solution with a volume ratio of 15% to reduce the pH value to 1.0 and then the third time CE analysis was conducted. Finally, 1 M NaOH was added to the L-tryptophan-HSA solution to readjust the pH value back to 7.4 for the fourth time CE analysis.

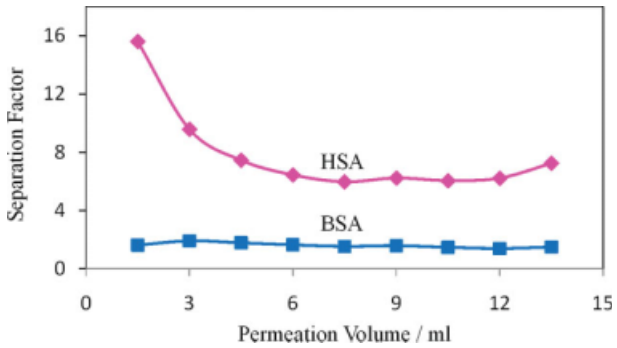

Figure 1. Comparison of tryptophan separation performance between BSA and HSA (Mem-5k was used for UF).

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Results and Discussion

Comparison of tryptophan separation performance between BSA and HSA

It can be seen in Figure 1 that the separation factor of D,L-tryptophan racemic mixture shows a large difference between BSA and HSA as stereoselective ligands when Mem-5k was used in this work. For HSA, the separation factor of tryptophan exhibits a highest value of 16 at the beginning and then reaches a plateau at the value of 6.5, whereas for BSA, the separation factor of tryptophan almost keeps unchanged at a value of 1.6. One possible reason is that the two enantiomers of D,L-tryptophan have different association constants to BSA and HSA.[30] HSA possesses a higher intrinsic enantioselectivity of 8.5 than that of 6.5 of BSA.[30]

Another possible and also most important reason is the difference in three-dimensional structure between these two kinds of serum albumins although their molecular weights (MWs) are very similar. As we know, BSA has a dimension of 14 $\times$ 4 $\times$ 4 nm,[32] and its hydrodynamic radius is 4.5 nm measured by dynamic light scattering,[33] whereas the shape of HSA in solution is roughly an equilateral triangle with a side of 8 nm and a thickness of 3 nm and its hydrodynamic radius is around 7–8 nm.[34] When the membrane pore has a size between 4 and 8 nm, it may completely constrain HSA transport from three directions, whereas for BSA transport, only one direction is restricted. Therefore, it is easier for BSA to pass through the membrane than HSA no matter in terms of their differences in hydrodynamic radius or in crystalline structure. Figure 2 may verify this point through a comparison of capillary electropherograms of samples collected at the permeate side of Mem-5k between BSA and HSA as stereoselective ligands. It is obvious that the permeate solution still contains the protein molecules when BSA is used as a ligand, and in contrast, no protein molecules can be detected in the permeate solution when HSA is used as a ligand.

To prevent the overlap of D-tryptophan and L-tryptophan peaks in electropherograms, the pH value of permeate sample had to be kept under the acidic condition by adding a 1 M HCl/0.2 M KCl solution before it was injected into CE for concentration analysis. However, such a low pH will induce tryptophan to lose the binding ability to the positively charged BSA,[35] and thus leading to a significant

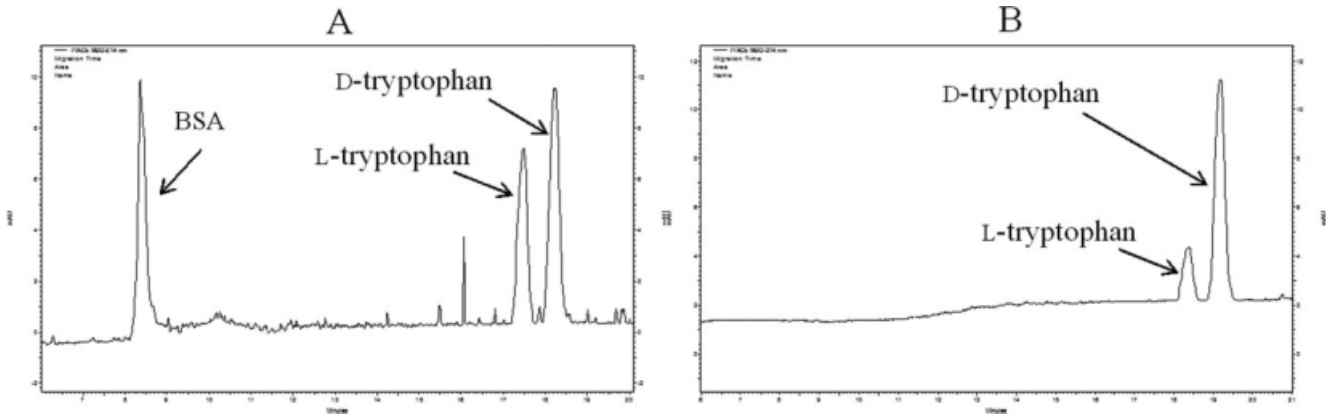

Figure 2. Comparison of capillary electropherograms of permeate samples via Mem-5k between BSA and has.

(A) BSA as the ligand and (B) HSA as the ligand.

enhancement in the concentration of free L-tryptophan. When Mem-5k is used, the BSA-L-tryptophan complexes appear in the permeate solution during the filtration because BSA has an irregular 3D structure as mentioned earlier. Later, those initially bound L-tryptophan molecules will be discharged from BSA-L-tryptophan complexes due to CE analyzing conditions (i.e. pH $\sim$ 2), thereby resulting in a poor D/L-tryptophan separation performance. This also explains why the yield of L-tryptophan using BSA as the stereoselective ligand is much higher than that using HSA as the stereoselective ligand as illustrated in Figure 3 although the same membrane (i.e. Mem-5k) is used in these two cases.

Effect of membrane pore sizes on tryptophan separation performance

A comparison of the yields of L-tryptophan and D-tryptophan through PES UF membranes with different pore sizes is illustrated in Figure 4 when HSA is used as the stereoselective ligand in this work. This figure reveals that the yield of tryptophan enantiomers increases in the initial stage; then reaches a relative plateau in the middle stage and starts to decrease in the final stage. Although the magnitude of yield and their variations as a function of time is different for PES membranes with different pore sizes, the general trend is the same, implying a similar permeation mechanism. The reason that tryptophan enantiomers shows a lower yield at the beginning is possibly due to the fact that tryptophan molecules need time to overcome the effect of hydrophobic adsorption between them and membrane pore wall on their transport across the membrane. The decrease in tryptophan yield in the final stage may be because the membrane pore size is narrowed to an extent due to the significant adsorption of macroligand, HSA, so that tryptophan transport across the membrane is restrained.

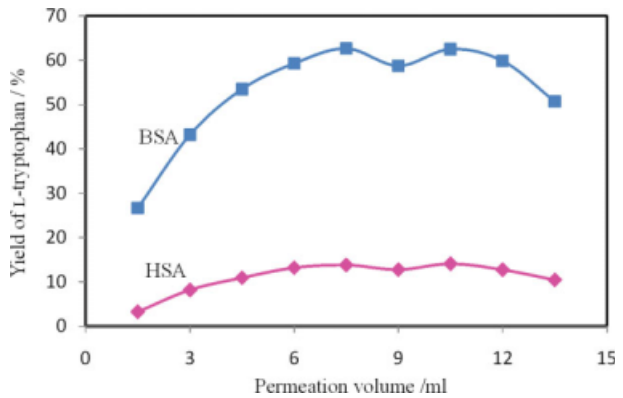

Figure 3. Comparison of yields of L-tryptophan between BSA and HSA in the permeate side (Mem-5k was used for UF).

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

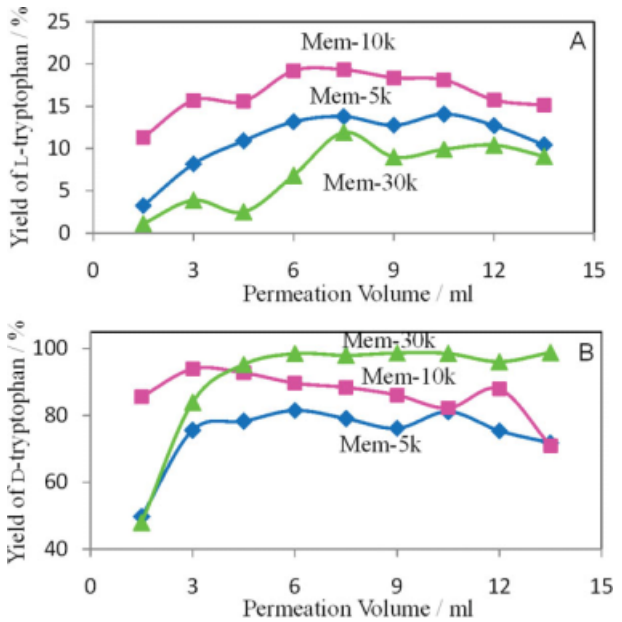

Figure 4. Comparison of yields of L- and D-tryptophan of PES UF membranes with different pore sizes in the permeate side (HSA as the stereoselective ligand).

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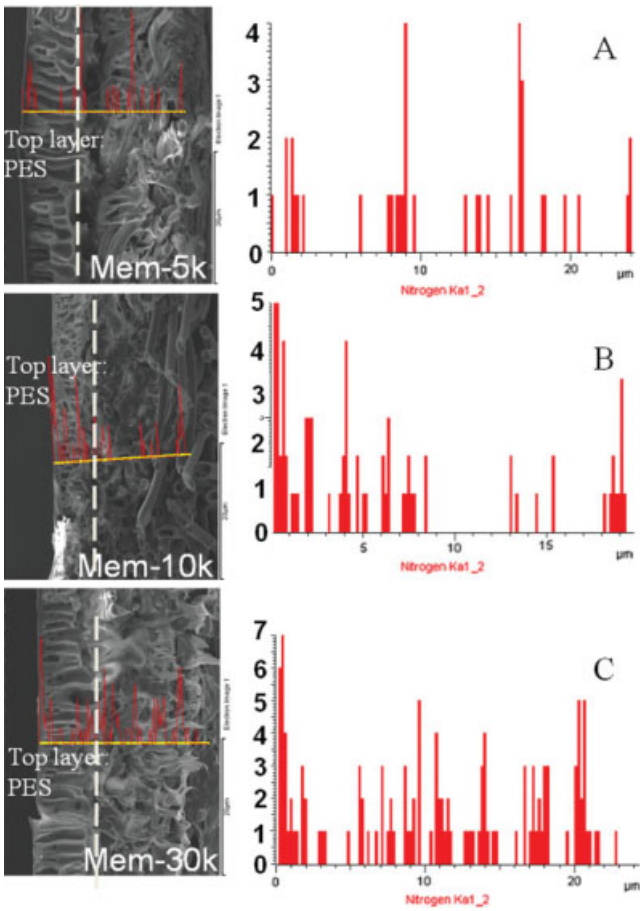


Figure 5. The distribution profile of nitrogen element along the cross-section of PES UF membranes with different pore sizes analyzed by EDX.

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Another point worthy of notice is that the yield of D-enantiomer increases as an order of Mem-5k < Mem-10k < Mem-30k, whereas L-enantiomer displays a lowest yield when Mem-30k is used. The changing trend of D-enantiomer yield is easily understandable because the membrane pore size is a dominant factor in the event that other factors such as ligand binding are less involved in D-tryptophan transport. However, the occurrence of the lowest yield of L-enantiomer at Mem-30k seems counter-intuitive at first because Mem-30k possesses the largest pore size among these three types of membranes.

The distribution of stereoselective ligand, HSA, within the membrane cross-section may offer a clue to explore the mechanism behind this abnormal phenomenon. An experiment was designed to trace the HSA distribution by filtrating the pure HSA solution across the membrane using a same concentration and pressure as tryptophan separation experiments and then characterizing the distribution profile of nitrogen element along the membrane cross-section via EDX to represent the state of HSA molecules due to the absence of nitrogen in PES membrane materials. It can be seen in Figure 5 that Mem-5k shows the lowest intensity and width in nitrogen distribution because its small pore size blocks the transport of most HSA molecules. With increasing the membrane pore size, a relatively intense and wider nitrogen distribution profile appears at Mem-30k, which suggests more HSA molecules may be retained within the cross-section of Mem-30k because of a fine match between the regular structure of HSA molecules (i.e. equilateral triangle) and suitable membrane pore size. Therefore, the retention of HSA molecules offers a chance that free L-tryptophan molecules may experience a second stage binding within the membrane cross-section when they diffuse through the membrane as illustrated in Figure 6. Although the number of HSA molecules within the membrane cross-section is not as many as the feed solution, the contact probability between the binding site of HSA molecules and free L-tryptophan may be significantly enhanced since they are restricted together inside a narrow space (i.e. membrane pores). This may be the reason why Mem-30k shows the lowest yield of L-tryptophan in this work.

Therefore, it is explainable that under the steady state, Mem-5k and Mem-10k shows a similar separation factor of 6.5 as illustrated in Figure 7 because they reflect an intrinsic separation factor of HSA as a stereoselective ligand. However, when Mem-30k is used, its suitable pore size retains a part of HSA molecules within the membrane cross-section, thus providing the second stage binding opportunity for free L-tryptophan molecules therein. As a result, under the steady state, Mem-30k exhibits a separation factor of 12-15 as shown in Figure 7, which is even higher than the intrinsic value of HSA. A simultaneous enhancement in separation factor and D-tryptophan yield makes the affinity UF system developed by a combination of Mem-30k and HSA ligand in this work very attractive for industrial applications in chiral separation.

Regeneration of HSA

To further improve the applicability of affinity UF system developed in this work, the regeneration of stereoselective ligand, HSA, is an issue necessary to be addressed to save the material cost. Therefore, an experiment was designed by

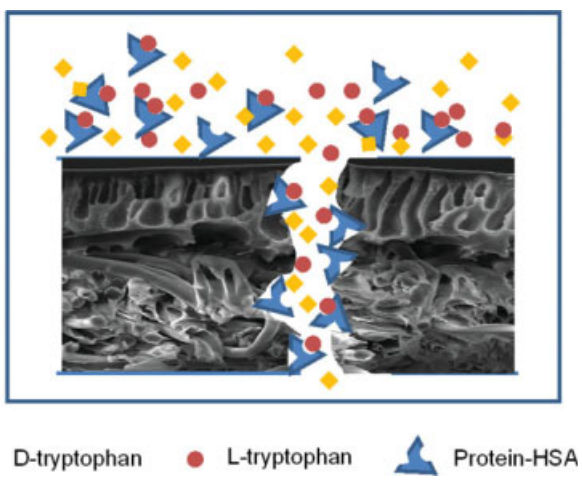


Figure 6. The schematic of the second stage binding between retained HSA molecules and free L-tryptophan within the cross-section of Mem-30k.

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

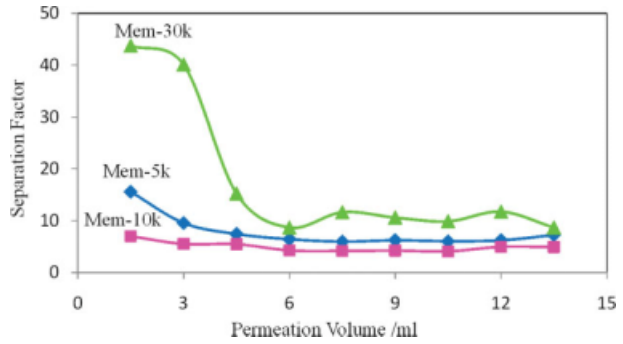


Figure 7. Comparison of tryptophan separation factor of PES UF membranes with different pore sizes (HSA as the stereoselective ligand).

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

a pH adjustment as illustrated in Figure 8 to demonstrate the feasibility of HSA regeneration after D,L-tryptophan separation. First, through comparison with Figure 8A, Figure 8B reveals the disappearance of L-tryptophan peak and the appearance of one new peak at $\sim$ 9 min after the addition of HSA. These changes hint that almost all the L-tryptophan molecules are bound by HSA at pH = 7.4, thus forming an L-tryptophan–HSA complex.

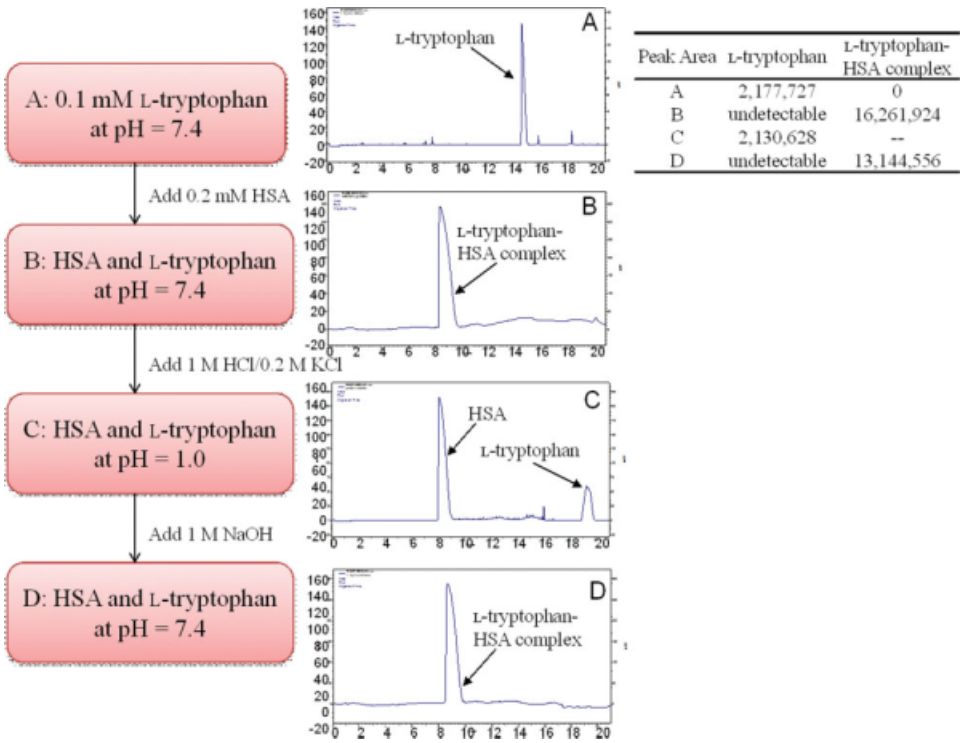
Second, when the pH value of solution is reduced to 1.0 by the addition of inorganic acid, one peak in Figure 8B is split into two peaks in Figure 8C, wherein one represents HSA and the other expresses L-tryptophan via identification. A difference in position of L-tryptophan peak between Figures 8A and C is probably due to a difference in pH value between these two stages, thus affecting the elution time of L-tryptophan during CE testing. Through comparing the peak area of L-tryptophan between Figures 8A and C, a conclusion can be achieved that almost all the bound L-tryptophan molecules are released from positively charged HSA when the pH value is decreased to 1.0. This discovery offers an opportunity to separate L-tryptophan from HSA by using size-exclusion membranes at the acidic condition, thus obtaining high-purity L-tryptophan at the permeate side and meanwhile, recovering HSA at the retentate side.

Finally, when the pH value of solution is adjusted back to 7.4 by the addition of sodium hydroxide, the second time disappearance of L-tryptophan peak suggests the formation of L-tryptophan–HSA complex again. This observation documents that HSA recovered under the acidic condition can still exhibit a high binding ability to L-tryptophan as long as the solution is adjusted back to the neutral condition. Therefore, a series of above-mentioned experimental results demonstrate the feasibility of HSA regeneration via the simple pH adjustment after D,L-tryptophan racemic mixture separation.

Conclusions

The following conclusions can be drawn from this work:

As a stereoselective ligand, HSA displays a much higher D,L-tryptophan selectivity of 6.5 than that of 1.6 of BSA in an affinity UF system, although their intrinsic D,L-tryptophan selectivity is similar. The most possible reason is that it is



Peak Area	L-tryptophan	L-tryptophan-HSA complex
A	2,177,727	0
B	undetectable	16,261,924
C	2,130,628	--
D	undetectable	13,144,556

Figure 8. Flowchart of regeneration of HSA ligand and comparison of electropherograms of solution samples at corresponding stages.

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

more difficult for Mem-5k to hold BSA molecules at the retentate side due to its irregular crystalline structure and smaller hydrodynamic radius than HSA molecules. The appearance of BSA molecules in the permeate solution may cause a severe harm for tryptophan separation performance because those initially bound L-tryptophan molecules are released from BSA under CE testing conditions, thus resulting in a significant increment in free L-tryptophan concentration.

The yield of D-tryptophan increases as an order of Mem-5k < Mem-10k < Mem-30k which is attributed to the effect of membrane pore sizes; however, L-tryptophan exhibits the lowest yield when Mem-30k is used. This may be due to the fact that a fine match between the regular crystalline structure of HSA molecules and suitable pore size of Mem-30k makes some HSA molecules be retained within the membrane cross-section, thus offering a second stage binding opportunity for free L-tryptophan molecules. As a result, in the Mem-30k system, HSA exhibits a D,L-tryptophan selectivity of 12-15 under the steady state which is even higher than its intrinsic value.

The feasibility of HSA regeneration after D,L-tryptophan separation has been demonstrated via a simple pH adjustment. Most of the bound L-tryptophan molecules are released from positively charged HSA when the pH value is adjusted to an acidic condition, hence providing an opportunity to separate L-tryptophan from HSA. In addition, the recovered HSA molecules can still exhibit a high binding ability to L-tryptophan when the pH value is adjusted back to the neutral condition.

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