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Chiral separation of DL-tryptophan using porous membranes containing multilayered bovine serum albumin crosslinked with glutaraldehyde

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Abstract

Bovine serum albumin (BSA) as a chiral ligand was captured uniformly throughout a porous hollow-fiber membrane at a level of 160 mg/g by the polymer chains grafted onto the membrane. BSA was bound in three layers with an end-on orientation to diethylamino groups on the graft chains which expanded from the pore surface towards the pore interior due to mutual electrostatic repulsion. Subsequently, crosslinking of BSA with a 0.025% (w/w) of glutaraldehyde in a Tris–HCl buffer (pH 8) for 4 h was effective in stabilizing the amount of BSA immobilized at a level of 150 mg/g. A solution of DL-tryptophan in a Tris–HCl buffer as a mobile phase permeated the crosslinked-BSA multilayered membrane and produced a chromatogram with a separation factor of 12. BSA leakage was not detected in the mobile phases at various pH values and organic modifiers. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Immobilized proteins; Chiral stationary phases, LC; Membranes; Albumin; Glutaraldehyde; Tryptophan; Amino acids

1. Introduction

Some proteins such as bovine serum albumin (BSA) [1] and ovoglycoprotein [2] have recognition sites for enantiomers of compounds such as amino acid derivatives and drugs. The immobilization of BSA in matrices is effective as a means to separate chiral molecules because the necessity of recovering BSA after contact with the analyte is eliminated. Previous studies on immobilized BSA are summarized in Table 1 [3–14]. Among these studies, Hofstetter et al. [12] adopted perfusion beads as a matrix and demonstrated a high rate of chiral separation of amino acid derivatives because of convective transport of enantiomers to the recognition sites. The use of porous hollow-fiber membranes as a matrix is advantageous over beads in that linear scale-up is possible [15].

In our previous publications, ion-exchange-group-containing polymer chains were grafted onto porous hollow-fiber membranes uniformly throughout the membrane [16]. BSA was captured in multilayers by the graft chains based on the ion-exchange inter-

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Table 1
Previous studies on BSA chiral stationary phase

Shape	Matrix	Binding mode	Binding amount of BSA (mg/g)	Target analyte	Ref.
Bead	Agarose	Covalent	42 mg/column	Trp	[3]
	Silica	Covalent	–	Trp, Bz-Ala, kynurenine warfarin, mandelic acid	[4]
	Silica	Covalent	144	Trp, lorazepam, etc.	[5]
	Silica	Covalent	36 mg/column	Trp, Bz-Ala, 2-phenylbutyric acid	[6]
	HEMA polymer	Covalent	–	Trp, Bz-Ala kynurenine, etc.	[7]
	Silica	Covalent	127	Benzoin, warfarin oxazepam, etc.	[8]
	Silica	Covalent	66	Trp, kynurenine	[9]
	Silica	Covalent	33 (HSA)	Trp	[10]
	Silica	Physical adsorption	109	Amino acid derivatives	[11]
		Covalent (with CDI)	110		
		Covalent (with GA)	114		
	PS–DVB perfusion bead POROS	Covalent	257	Amino acid derivatives, ibuprofen, kynurenine	[12]
Membrane	Polysulfone UF	Covalent	–	Phe, Leu, Trp	[13]
	Polyethylene MF hollow-fiber	Ion-exchange adsorption	49–490	–	[14]
	Polyethylene MF hollow-fiber	Ion-exchange adsorption and crosslinking with Ga	150	Trp	This work

Abbreviations: BSA: Bovine serum albumin; HSA: human serum albumin; HEMA: hydroxyethylmethacrylate; PS–DVB: polystyrene–divinylbenzene; UF: ultrafiltration; MF: microfiltration; CDI: 1,1-carbonyl diimidazole; GA: glutaraldehyde; Trp: tryptophan; Bz-Ala: *N*-benzoylalanine; Phe: phenylalanine; Leu: leucine.

action during permeation by BSA in a buffer [17]. When DL-tryptophan was added to a buffered mobile phase that permeated the BSA-multilayered porous membrane, L-tryptophan was retained with greater selectivity than D-tryptophan, resulting in a chiral resolution with a separation factor of about ten [18]. Here, the use of a common buffer for BSA binding and subsequent enantiomer injection was required to ensure a negligible leakage of BSA. This restriction in the selection of the mobile phase should be overcome to improve the performance of the chiral separation.

The surface of BSA contains amino groups ($-NH_2$) and carboxyl groups (-COOH) originating from constituent amino acids; therefore, crosslinking of BSA with an appropriate crosslinker will be effective to prevent BSA leakage in various mobile phases (Fig. 1).

The objectives of this study were twofold: (1) to crosslink BSA molecules multilayered in polymer chains grafted onto a porous membrane, and (2) to examine the effect of crosslinking on chiral separation by the resultant membrane.

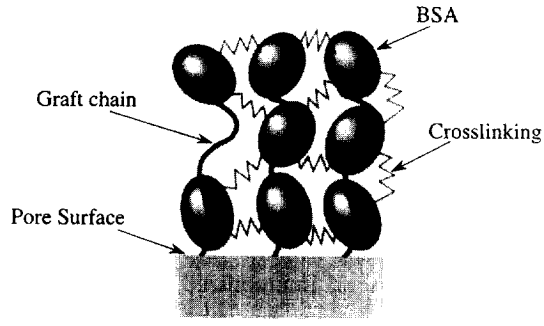

Fig. 1. Crosslinking of BSA captured by the graft chains.

2. Experimental

2.1. Materials

A porous hollow-fiber membrane made of polyethylene, which was supplied by Asahi Chemical Industry, Japan, was used as the trunk polymer for grafting. BSA was purchased from Sigma (St. Louis, MO, USA). An aqueous solution of 25% (w/w) glutaraldehyde was from Nacalai Tesque (Kyoto, Japan). Other chemicals were of reagent grade.

2.2. Multilayering of BSA onto a porous membrane

An anion-exchange-group-containing porous hollow-fiber membrane was prepared according to Tsuneda et al. [14]. An epoxy-group-containing monomer (glycidyl methacrylate, GMA) was grafted onto an electron-beam-irradiated porous polyethylene (PE) hollow-fiber membrane. The amount of GMA grafted onto the PE membrane was 1.9-fold the mass of the PE membrane. Subsequently, some epoxy groups were converted to a diethylamino group as an anion-exchange group by reaction with diethylamine (DEA) and the remaining epoxy groups reacted with ethanolamine (EA) to retard nonselective adsorption of proteins. The resultant membrane is referred to as a DEA–EA fiber. Properties of the DEA–EA fiber are summarized in Table 2.

Table 2
Properties of the DEA–EA membrane

Dimensions in wet state	
Inner diameter (mm)	2.4
Outer diameter (mm)	4.4
Density (g/ml)	0.4
DEA group density (mol/kg[a])	2.3
Specific surface area[b] (m^2/g)	5.5
Theoretical monolayer binding capacities of BSA	
End-on (mg/g)	44
Side-on (mg/g)	13

[a] Kilograms of dry state of DEA–EA membrane.
[b] BET surface area.

A hollow fiber 5 cm long was positioned in an I-configuration. A solution of 2.0 mg/ml BSA in a Tris–HCl buffer (pH 8.0) was permeated through the hollow fiber radially from the inside to the outside at a constant flow-rate of 1.5 ml/min. The BSA concentration of the effluent penetrating the outside surface of the hollow fiber was monitored continuously by measuring its UV absorbance at 280 nm. After equilibrium was attained, the feed was switched to the buffer solution to wash the pores. The amount of BSA captured by the graft chains was calculated as follows:

$$q = \int_0^{V_s} (C_0 - C)\mathrm{d}V/W \quad (1)$$

where C_0 and C are the BSA concentrations of the feed and effluent, respectively. The terms V, V_s, and W are the effluent volume, the effluent volume when C reaches C_0, and the mass of the DEA–EA fiber, respectively. The degree of multilayer binding was defined by dividing the value of q by a theoretical monolayer binding capacity assuming that a BSA molecule was bound with an end-on orientation. The resultant BSA-multilayered porous hollow-fiber membrane is referred to as a BSA fiber.

2.3. Crosslinking of BSA captured by graft chains

The BSA fiber was immersed in a 0.025% (w/w) solution of glutaraldehyde in a Tris–HCl buffer at 303 K. The molar ratio of glutaraldehyde dissolved in the solution to BSA captured by the graft chains was set at 70. The reaction time ranged from 0.05 to 72 h. After the crosslinking reaction, 0.5 *M* NaCl was forced to permeate the fiber. The amount of BSA immobilized by crosslinking was defined by subtracting the amount of BSA eluted with 0.5 *M* NaCl from the amount of BSA previously captured by the graft chains. The resultant fiber is referred to as a BSA–CL(X) fiber, where CL and X designate crosslinking and crosslinking time in hours, respectively. To determine the distribution of BSA across the membrane thickness of the BSA–CL(4) fiber, sulfur distribution was observed by an X-ray microanalyzer (XMA) after the fiber was dried under reduced pressure.

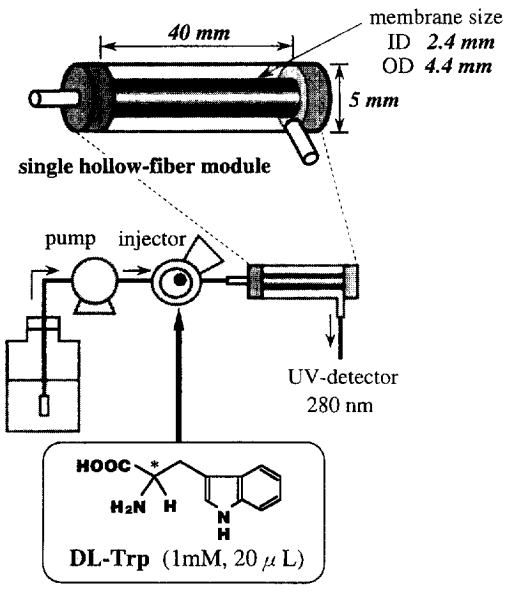

Fig. 2. Experimental apparatus for chiral resolution.

2.4. Chiral separation of DL-tryptophan

A schematic illustration of a single fiber module is shown in Fig. 2. The inner diameter and length of the polyethylene tube were 5.0 mm and 5 cm, respectively. A 5-cm long BSA–CL(X) fiber was housed in the tube and plugged at both ends using a 5-mm-thick epoxy resin. The module was mounted in a liquid chromatograph (Hitachi L-7000). A solution of 20 μl of 1.0 mM DL-tryptophan was injected into the mobile phase. The following buffers, as mobile phases, permeated through the module at a flow-rate of 1.5 ml/min: a 20 mM Tris–HCl buffer (pH 8.0) and a 10 or 20 mM phosphate buffer, the pH of which ranged from 5 to 9, and 5% (v/v) of 2-propanol, methanol or acetone in the phosphate buffer (pH 8.3). DL-Tryptophan was determined by measuring the UV absorbance at 280 nm or 210 nm. The experiment on chiral separation was performed at 296 and 298 K.

The separation factor was calculated from the resultant chromatogram as follows:

$$\text{separation factor} = (t_L - t_0)/(t_D - t_0) \quad (2)$$

where t_D and t_L are the retention times of D- and L-tryptophan, respectively. t_0 is the void time.

3. Results and discussion

3.1. Crosslinking of BSA captured by graft chains

The amount of BSA captured by the anion-exchange-group-containing polymer chains grafted onto the porous hollow-fiber membrane was 160 mg/g, which was equivalent to a degree of multilayer binding of three. Expansion of the graft chains due to mutual electrostatic repulsion provided three-dimensional binding sites for BSA [14].

The amount of BSA immobilized after crosslinking with glutaraldehyde is shown in Fig. 3 as a function of crosslinking time. The amount of BSA immobilized increased with an increasing crosslinking time and leveled off at 150 mg/g over 4 h.

A uniform distribution of sulfur was observed across the BSA–CL(4) fiber wall by XMA, as shown in Fig. 4 where the y-axis shows the intensity of the X-ray characteristics of sulfur. This indicates that BSA was immobilized uniformly across the 1.0-mm thick hollow-fiber membrane via ion-exchange adsorption and subsequent crosslinking.

3.2. Chiral separation using crosslinked-BSA multilayered porous membranes

A DL-tryptophan in the Tris–HCl buffer was injected into the same buffer and permeated through the BSA–CL(X) fiber. An example of the chromatogram of the BSA–CL(1) fiber for DL-tryptophan is shown in Fig. 5. The separation factor is shown in Fig. 6 as a function of X, where the crosslinking time X ranged from 0.05 to 72 h. The result for the

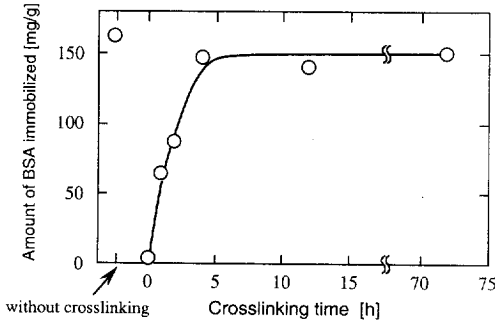

Fig. 3. Immobilization of BSA as a function of crosslinking time.

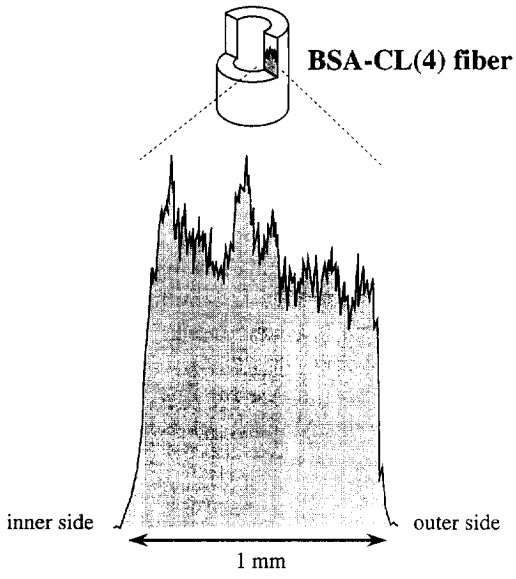

Fig. 4. XMA profile of sulfur across the BSA–CL(4) fiber.

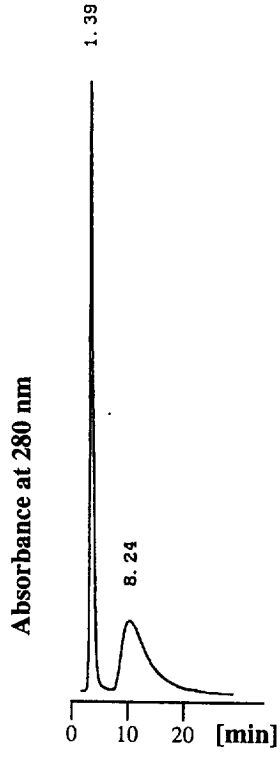

Fig. 5. An example of a chromatogram of the BSA–CL(1) fiber for DL-tryptophan. Mobile phase, 10 m*M* phosphate buffer (pH 8.0); flow-rate, 1.5 m/min; temperature, 298 K.

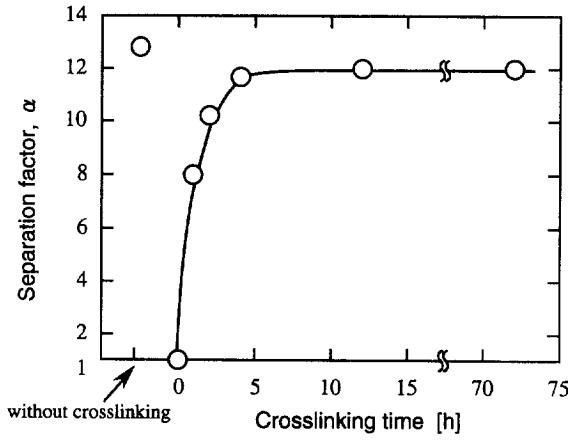

Fig. 6. Effect of crosslinking time on separation factor. Mobile phase, 20 m*M* Tris–HCl buffer (pH 8.0); temperature, 298 K.

noncrosslinked BSA fiber, i.e., the BSA fiber, is also shown in this figure, where BSA was not eluted with 0.5 *M* NaCl. The separation factor of the BSA–CL(*X*) fiber increased with an increasing amount of immobilized BSA, and reached a maximum of 12, which was 93% that of the BSA fiber. As a result, crosslinking with 0.025% (w/w) glutaraldehyde aqueous solution at a molar ratio of glutaraldehyde to BSA of 70 maintained the chiral separation performance of BSA. The possibility that the remaining end group of glutaraldehyde reacts with the amino acid of tryptophan was neglected because the separation factor was reproducible for repeated injection of DL-tryptophan.

3.3. Stability of the BSA–CL fiber

The phosphate buffers at various pH values were used as mobile phases for the chiral separation of DL-tryptophan. The separation factor is shown in Fig. 7 as a function of pH. The pH ranged from 5 to 9 to avoid the deterioration of BSA. The separation factor increased with increasing pH. The highest separation factor of eight for the BSA–CL(1) fiber in the phosphate buffer was obtained at pH 9. This pH dependence of the separation factor agreed well with the results reported with BSA immobilized on silica [4] and HEMA polymer [7]. Since the pH of the buffer suitable for the adsorption of BSA onto the graft chains based on an ion-exchange interaction

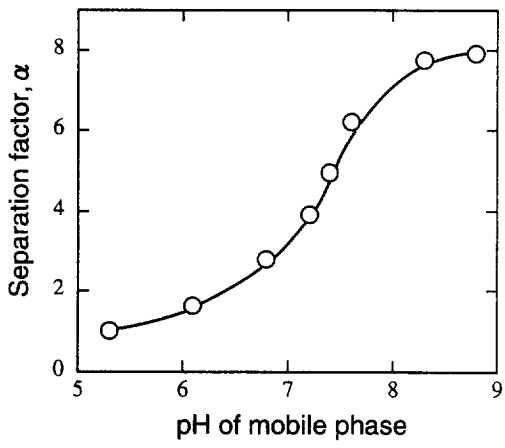

Fig. 7. pH dependence of separation factor. Fiber, BSA–CL(1); mobile phase, 20 m*M* phosphate buffer (pH 8.0); temperature, 296 K.

does not always agree with the optimum pH for chiral recognition, crosslinking of BSA captured by the graft chains is advantageous.

Appropriate organic solvents, i.e., organic modifiers, are added to shorten the retention time of the chromatogram. The separation factors obtained from alternative injections of the buffer and the buffers containing 5% (v/v) various modifiers such as 2-propanol, methanol, and acetone were reversible. This is indicative of a high stability of the BSA–CL(4) fiber as a stationary phase.

4. Conclusions

BSA was captured by polymer chains grafted onto a porous hollow-fiber membrane uniformly throughout the membrane by an anion-exchange interaction. The amount of BSA captured was equivalent to three times the monolayer binding capacity of BSA on the pore surface. Subsequently, BSA was crosslinked with glutaraldehyde to avoid BSA leakage from the graft chains in various mobile phases. An optimum crosslinking time was selected to immobilize a maximum amount of BSA as a chiral ligand. DL-tryptophan added to a Tris–HCl buffer (pH 8.0) as a mobile phase permeated the crosslinked-BSA-immobilized porous hollow fiber resulting in a separation factor of 12. Because of its stability, BSA-immobilized hollow fiber is applicable for the chiral separation of enantiomers in a mobile phase with various pH values and organic modifiers.

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