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Silica sol–gel/organic hybrid material for protein encapsulated column of capillary electrochromatography

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Abstract

A new-type of sol–gel/organic hybrid composite material using gelatin or chitosan with tetramethoxysilane was developed for the bovine serum albumin (BSA)-encapsulated monolithic column for capillary electrochromatography (CEC). The composite monolith was used to immobilize BSA in a fused-silica capillary. The addition of gelatin and chitosan to the alkoxysilane enabled the enantioseparation of Trp. A very small amount of these polymers were effective for the enantioseparation. Especially, the monolithic column prepared from chitosan with tetramethoxysilane showed a high enantioselectivity for Trp enantiomers and the value ($\alpha' = t_2/t_1$, t_1: fast eluted enantiomer, t_2: second eluted enantiomer) reached 1.15 on CEC mode. Furthermore, the composite materials exhibited a higher stability compared to the silica sol–gel column. These results showed that the sol–gel/organic hybrid composite was useful as a monolithic matrix for the BSA-encapsulated column for CEC.
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1. Introduction

Chiral separations using proteins as chiral selectors are widely employed for the assay of drug enantiomers [1]. Because the binding of a drug is a part of its physiological role, bovine serum albumin (BSA) [2–6] and human serum albumin (HSA) [7–10] have been thoroughly studied regarding the characteristics of their binding sites. The resulting knowledge has been applied to the chiral separation of various drugs.

Capillary electrochromatography (CEC) has been regarded as a very promising analytical separation technique that combines the efficiency of capillary zone electrophoresis and the selectivity of liquid chromatography (LC) with the use of a solid stationary phase [11,12]. To take advantage of the prominent selectivity of proteins, CEC is thought to be an ideal technique.

Several techniques have been reported that use proteins for chiral separation in CEC. At present, there are mainly three techniques to immobilize proteins on a CEC column, that is, covalent binding to silica particles [10,13], cross-linked to a glutaraldehyde gel [4], and physical adsorption onto the capillary wall [9]. Recently, we developed a novel protein-encapsulation technique by the sol–gel method for the preparation of monolithic columns of CEC [14–16]. Using this technique, a BSA-encapsulated column was prepared using tetramethoxysilane (TMOS) with 10% methyltrimethoxysilane (MTMS) as the starting monomer. The alkoxysilane-based hydrogel could retain BSA in a capillary without losing its enantioselectivity, and Trp enantiomers were separated. Although this silica sol–gel possesses chemical inertness, and negligible swelling in aqueous and organic solutions, the brittleness of the silica sol–gel matrix is generally a major obstacle. To overcome these problems, we developed silica sol–gel based inorganic–organic hybrid materials for immobilization of BSA in a capillary. Two biopolymers; protein, gelatin, and polysaccharide, chitosan were used. Both biopolymers have

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been reported to be biocompatible and non-immunogenic [17,18], and have been considered for various applications [19].

In this study, protein-encapsulated monolithic columns were fabricated using natural polymers with TMOS for protein encapsulation.

2. Experimental

2.1. Materials and chemicals

Fused sillica capillary (75 μm i.d. × 375 μm o.d.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). TMOS, methacryloxypropyltrimethoxysilane (MPTMS), and chitosan 500 (300–700 cP) were purchased from Tokyo Kasei (Tokyo, Japan). DL-Trp was purchased from Sigma–Aldrich (Milwaukee, WI, USA). BSA (crystallized cold alchohol precipitate 97%) was purchased from Wako (Osaka, Japan). Gelatin was purchased from Kaken (Tokyo, Japan). Water was purified by a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. Monolithic capillary column preparation

The capillary column (30 cm) was pretreated with MPTMS as described in our previous reports [14]. The polyimide coating of the treated capillary was etched with concentrated sulfuric acid to make a detection window.

The sol–gel reaction was performed as described in our previous report [14–16]. The monomer solution was obtained by mixing the following reagents just prior to use: (1) 761 μL TMOS, (2) 169 μL water and (3) 11 μL 0.04 M HCl. This solution was stirred for 20 min to allow the formation of fully or partially hydrolyzed silane, $SiOH_{4-n}$ $(OMe)_n$.

Mixture solution of 6.25% BSA in 50 mM phosphate buffers (pH 5.0) and gelatin or chitosan solution was then added to this monomer solution. After mixing and ultrasonication for 5 s, the mixture solution was carefully aspirated with a 1.0-mL disposable syringe from the inlet of the capillary, which was in advance filled with 10 mM phosphate buffer (pH 7.0), until the sol plug becomes about 8-cm long under the microscopic observation. The ends of the capillary were then sealed with parafilm and placed at room temperature for at least 2 days to allow the gel formation.

After the gel was formed, the capillary was cut up to adequate length for instruments. The total length, effective length, and packed length were 28, 20, and 5 cm, respectively. The capillary was carefully installed in a CE cartridge and conditioned electrokinetically (−2 kV) with 10 mM phosphate buffer (pH 7.0) for 1 h to eliminate the all methanol or proteins which were not encapsulated in the sol–gel matrix.

2.3. CEC equipment

CEC experiment was carried out on a Beckman P/ACE5510 capillary electrophoresis instrument (Fullerton, CA, USA) with a diode array detector. Samples were introduced electrokinetically at the anodic side (4 kV, 5 s), and the same voltage was used for separation. The temperature was kept at 25 °C in all experiments.

All samples were prepared in the mobile phase, 20 mM phosphate buffer (pH 7.0). All solutions were filtered through a 0.22-μm membrane (Millipore) and degassed by ultrasonication. The mobile phase was 10 mM phosphate buffer (pH 7.0) for BSA-encapsulated column. The enantioselectivity was evaluated using α' (t_L/t_D), where t_D and t_L were the retention times of D-Trp and L-Trp, respectively, because of the following reasons; (1) it is hard to measure exact t_0, (2) the peak shape of L-Trp was often very asymmetric owing to the strong interaction with BSA, and it was difficult to calculate half width of the peak.

3. Results and discussion

3.1. Gelatin

Because it is known that gelatin undergoes a thermally reversible transition, the gelatin was initially used for the BSA-encapsulation without TMOS. The gelatin solution, which was mixed with BSA solution, was introduced into a capillary. Although a BSA-containing gel was formed within a capillary using high concentration of the gelatin solution (more than 300 mg/ml), the column does not separate DL-Trp, and the gel gradually eluted during use. Therefore, the hybrid material of gelatin and TMOS was used for the BSA-encapsulation.

The gelatin solutions at concentrations of 0.75, 1.5, and 3 mg/ml were added to the TMOS-hydrolyzed solution, and the BSA-encapsulated columns were prepared. A solution of 10 mM phosphate buffer (pH 7.0) was used as the elution buffer. The columns prepared using gelatin of more than 3 mg/mL were not stable in terms of current and could not resolve the Trp enantiomer. Although the peak was somewhat broad, the columns using 0.75 and 1.5 mg/mL gelatins could resolve the enantiomer (Fig. 1). D-Trp was eluted faster than L-Trp, and this elution order was the same as that described in previous reports [14–16]. This result indicates that encapusulated BSA using gelatin/TMOS retained the ability for enantioseparation. Although gelatin contains chiral centers, the content of gelatin was much smaller than those of BSA. Therefore, it was assumed that the enantioselectivity by gelatin was negligible. Both columns showed a similar enantioselectivity, and the α' value was 1.09 (0.75 mg/mL gelatin) and 1.08 (1.5 mg/mL gelatin) (Table 1). On the other hand, the retention time changed with a change in the concentration of the gelatin. The theoretical plate number of thiourea was 72,000 (N/m) (1.5 mg/mL gelatin). A small

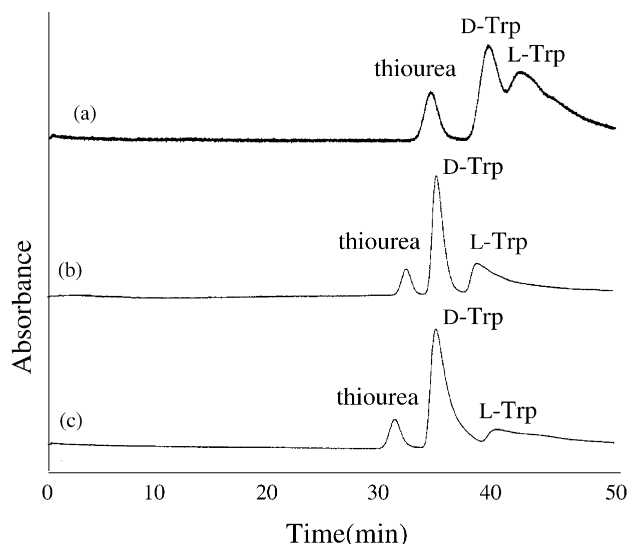

Fig. 1. Electrochromatograms of DL-Trp and thiourea using BSA-encapsulated monolithic column prepared using different compositions. Conditions: column: (a) 1.5 mg/ml gelatin, (b) 0.75 mg/ml chitosan, (c) 1.5 mg/ml chitosan. Sample: 11 mM DL-Trp and thiourea. Applied voltage: 2 kV for 3 s for injection, 2 kV for separation. Elution buffer: 10 M phosphate buffer (pH 7.0). Detection: 222 nm.

amount of gelatin was effective for the resolution. In a previous paper [14], it was reported that the enantiomeric separation of Trp was achieved by adding 10% (w/w) MTMS to TMOS. In case of gelatin, 0.3% (w/w) added to TMOS was sufficient. Because MTMS has a methoxy group, it forms silica networks when it was mixed with TMOS. The addition of MTMS to TMOS changes the chemical or physical characteristics of the network due to its methyl group, and this change was quite effective for the enantioseparation of Trp by BSA. On the other hand, a very small amount of gelatin was effective for the enantioseparation of Trp, which indicates that gelatin has an effect on the microenvironment around BSA, and not the gel structure. Because gelatin is a biopolymer, it was assumed that gelatin forms a bio-mimetic environment around the BSA.

3.2. Chitosan

Because the solubility of chitosan was very poor, a column using a high concentration of chitosan could not be prepared. Therefore, chitosan was added to TMOS for the preparation of the BSA-encapsulated column. Chitosan at concentrations of 1.5 and 0.75 mg/mL was used for the preparation of the BSA-encapsulated column. Both columns could separate the Trp enantiomers. Although chitosan contain chiral centers, the enantioselectivity by chitosan was also negligible just as the case in gelatin column. The column prepared with 1.5 mg/mL chitosan showed a better resolution. The α' value was 1.15, and this value was greater than that of the columns prepared from a mixed solution of gelatin/TMOS, and MTMS/TMOS (Fig. 1, Table 1). The value was similar to the previous reports that separated Trp enantiomers using BSA-immobilized column on CEC (1.05–1.08) and HPLC (1.07–2.21) [3–5]. Furthermore, the peak shape was better than that obtained by the gelatin/TMOS column. The theoretical plate number of thiourea was 130,000 (N/m) (0.75 mg/mL gelatin). Just like gelatin, a very small amount of chitosan was effective for the enantioseparation, and the enantioselectivity was superior to that of the MTMS/TMOS column. It is assumed that the abundantly present amino groups in the chitosan molecules provide a hydrophilic environment that is compatible with BSA. AFM and FT-IR studies of hybrid polymer of chitosan with (3-aoryloxypropyl) dimethoxymethylsilane showed that these polymer encapsulated protein with its native structure like a polymer made from only alkoxysilane did [20].

Table 1
Retention time and enantioselectivity of BSA encapsulated column

Additive	Concentration (mg/ml)	Retention time		Enantioselectivity (α')
		D-Trp	L-Trp	
Gelatin	0.75	30.99	33.85	1.09
	1.50	40.62	43.88	1.08
Chitosan	0.75	36.11	39.67	1.10
	1.50	36.01	41.38	1.15

The durability and repeatability of the column prepared using 0.75 mg/mL chitosan were examined. The R.S.D. of the retention times was 2.96% and 1.71% for D-Trp and L-Trp, respectively. The enantioselectivity was retained during 3 days of continuous use. These results were superior to those obtained by the MTMS/TMOS column, and presumably ascribed to the increased stability of the encapsulated BSA by the biological product, chitosan. A spectroscopic analysis is now progressing in our laboratory for clarifying the macro- or micro-structure of chitosan/TMOS, gelatin/TMOS, and MTMS/TMOS gels.

4. Conclusion

A BSA-encapsulated column for CEC was prepared using a natural polymer, gelatin and chitosan along with an alkoxysilane, TMOS. A very small amount of these polymers was effective for the enantioseparation of Trp. The resultant columns showed a good durability and enantioselectivity toward Trp. Especially, the column durability was improved by the addition of chitosan. These characteristics were superior to the columns prepared using only the alkoxysilane. These results indicate that the chitosan/TMOS column is promising as a protein-encapusulation column for CEC.

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