Preparation of Chiral Stationary Phase by Dynamic Grafting BSA on the Organic-Silica Hybrid Monolithic Column Based on Schiff-Base Reaction

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Abstract. The prepared hybrid monolithic column (HMC) with epoxy groups on the surface was reacted with polyethyleneimine, and then reacted with glutaraldehyde. The chiral monolithic column was prepared by dynamically grafting bovine serum albumin (BSA) onto the newly modified HMC by Schiff-base reaction. The obtained chiral monolithic column was characterized by SEM and IR. The effects of the concentration of the BSA solution, flow rate and grafting time on the grafting amount were optimized. The properties of chiral monolithic column were tested with benzoin as the chiral object. The results showed that the obtained grafting amount of BSA was 56.1 mg/g under the optimized conditions. The chiral monolithic column had homogeneous network skeleton structure and good reusability. The method of preparing chiral monolithic column had good reproducibility.

1. Introduction
About 50% of the drugs used in clinical practice are chiral drugs [1], and the efficacy of different enantiomers of chiral drugs is quite different, which may easily lead to side effects after taking many drugs and seriously threaten human life [2]. The joint commission on accreditation of international medical and health institutions (JCI) specifies that all new drugs marketed must account for the respective pharmacological effects, toxicity and effects of the enantiomers contained in the drug [3]. For the safety and accuracy of medication, efficient chiral separation methods have been the focus of research. Chromatographers have studied chiral selection agents such as macrocyclic antibiotics, polysaccharides, crown ethers and proteins from the perspective of chromatographic stationary phases [4]. The structure of a protein makes it possible to separate different enantiomers of a chiral compound and also makes it a good chiral selection reagent. The commonly used protein stationary phases are serum albumin, glycoprotein and enzymes. The commonly used methods for immobilizing proteins include Schiff-base method (reduction amination method), n-hydroxysuccinimide (NHS) method, carbonyl diimidazole (CDI) method, epoxy method, ethyl dimethyaminopropyl carbon diimide (EDC) method and toluenesulfonyl chloride method [5]. Among them, the Schiff-base method is used more [6].

In this work, based on the prepared hybrid monolithic column with a large-pore continuous network structure [7, 8], the bovine serum albumin (BSA) was grafted onto the newly modified hybrid monolithic column by the method of Schiff-base. The chiral monolithic column was characterized by scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR). The effects
of the concentration of the BSA solution, flow rate and grafting time on the grafting amount were optimized. The properties of chiral monolithic column were tested with benzoin as the chiral object.

2. Materials and Methods

2.1. Instrumental and Materials

Methyltrimethoxysilane (MTMS) was obtained from Nanjing Lianye Chemical Co. Ltd. (Shanghai, China). γ-(2,3-epoxypropyl)propyltrimethoxysilane (KH560) was obtained from Shanghai Bangcheng Chemical Co. Ltd. (Shanghai, China). Bovine serum albumin (BSA) was obtained from Beijing Quanshijin Biotechnology Co. Ltd. (Beijing, China). Polyethylenimine (PEI) was obtained from Shanghai Jizhi Biochemical Technology Co. Ltd. (Shanghai, China). All other chemicals were of analytical or HPLC grade. Double deionized water (DDW) was used in all the experiment. All solutions for HPLC were filtered through a 0.22 μm filter.

A Shimadzu LC-20A HPLC system (Kyoto, Japan) was used in this study. The solutions were pumped by LSP01-1BH high pressure injection pump (Lange, China).

2.2. Methods

The hybrid monolithic column (HMC) was prepared in a stainless steel column (50 mm × 4.6 mm). 2.0 mL MTMS, 0.75 mL methanol and 0.5 mL nitric acid (1.0 mol/L) were put into a conical flask. The solution was mixed and sonicated for 2 min, and then injected into the stainless steel column. The column was sealed at both ends. The stainless steel column was placed vertically for 24 h in a water bath at 40 °C. After washing with methanol, the HMC was dried under vacuum at 60 °C.

After washing with methanol, the HMC was activated with 0.1 mol/L nitric acid at a flow rate of 0.1 mL/min for 4 h, and finally the HMC was washed with DDW until the outflow was neutral. After washing with methanol for 10 min, the mixed solution of KH560 and methanol (3/2, v/v) was pumped into the activated HMC at a flow rate of 0.1 mL/min at 60 °C for 10 h. After the reaction was completed, the unreacted residue was removed by washing with methanol.

A 10% PEI aqueous solution was pumped into the monolithic column at a flow rate of 0.2 mL/min. After 10 min, the monolithic column was sealed, and then placed for 12 h in a water bath at 55 °C. 0.5 mol/L glutaraldehyde phosphate buffer was used to circulate in the monolithic column for 4 h at a flow rate of 0.1 mL/min to ensure the graft of aldehyde group. The monolithic column was washed with a phosphate buffer solution of pH 7.2 at a flow rate of 0.1 mL/min, and then the BSA solution of pH 7.2 was pumped into the functionalized HMC at a flow rate of 0.4 mL/min to prepare a chiral monolithic column. A 0.01 mol/L sodium cyanoborohydride solution was blocked in the monolithic column for 2 hours. Finally, the monolithic column was washed with DDW and 0.1 mol/L phosphate buffer solution of pH 7.2 until the pH was stable. The synthesis mechanism of chiral monolithic column was shown in figure 1.

![Figure 1. Synthesis mechanism of chiral monolithic column.](image-url)
3. Results and Discussion

3.1. Characterization of the Chiral Monolithic Column

The HMC, the monolithic column grafted KH560 (KH560-HMC), the monolithic column grafted PEI (PEI-HMC), and the monolithic column grafted BSA (BSA-HMC) were characterized by SEM. As shown in figure 2, the prepared monolithic columns had homogeneous network skeleton structure. The morphology of the monolithic column before and after the reaction did not change, showing that the structure of the monolithic column would not deform or collapse due to the pressure of the pump reaction, and its structure had certain stability.

![SEM images of monolithic columns](image)

**Figure 2.** SEM images of monolithic columns (a) HMC; (b) KH560-HMC; (c) PEI-HMC; (d) BSA-HMC.

The HMC, the monolithic column grafted KH560 (KH560-HMC), the monolithic column grafted PEI (PEI-HMC), the monolithic column grafted glutaraldehyde (CO-HMC) and the monolithic column grafted BSA (BSA-HMC) were characterized by IR, as shown in figure 3. In figure 3a, 1274 cm\(^{-1}\) was the deformation vibration peak of C-H in Si-CH\(_3\), near 2933.40 cm\(^{-1}\) was the asymmetric stretching vibration peak of C-H of -CH\(_2\)-, which was derived from the unhydrolyzed alkoxy group in the skeleton. Strong absorption peaks near 1125 cm\(^{-1}\) and 1030 cm\(^{-1}\) were attributed to Si-O-Si. 768 cm\(^{-1}\) was the vibrational absorption peak of Si-O. In figure 3b, the peak at 917 cm\(^{-1}\) was the characteristic absorption peak of the epoxy group, indicating the successful grafting of KH560. Comparing figure 3c with figure 3b, the absorption peak of the epoxy group disappeared, and the vibration peak of N-H appeared, indicating the successful grafting of PEI. In figure 3d, 1720 cm\(^{-1}\) was the characteristic peak of C=O. The N-H absorption peak at 3400 cm\(^{-1}\) in figure 3e was significantly enhanced, indicating that BSA was successfully covalently grafted onto the hybrid monolithic column.

![FT-IR spectra of monolithic columns](image)

**Figure 3.** FT-IR spectra of monolithic columns: (a) HMC; (b) KH560-HMC; (c) PEI-HMC; (d) CO-HMC; (e) BSA-HMC.
3.2. Optimization of Grafting Amount of BSA

The concentration of the BSA solution, flow rate and grafting time, which affected the grafting amount were optimized, as shown in figure 4. In the end, we chose to react a 6 mg/mL BSA solution at a flow rate of 0.4 mL/min for 6 h, and the obtained grafting amount was 56.1 mg/g.

![Figure 4](image)

**Figure 4.** Optimization of grafting amount of BSA molecule: (a) concentration of the BSA solution; (b) flow rate; (c) grafting time.

| Table 1. Reproducibility of benzoin separation by monolithic column. |
|-------------------------|------------------------|------------------------|------------------------|------------------------|
| Form                   | $k_1$                 | $k_2$                 | $\alpha$               | $Rs$                  |
|                        |                       |                       |                        |                        |
| Run-to-run ($n=10$)    | 9.67                  | 12.10                 | 1.25                   | 3.78                   |
|                        | 9.62                  | 12.02                 | 1.25                   | 3.74                   |
|                        | 9.57                  | 11.98                 | 1.25                   | 3.74                   |
|                        | 9.65                  | 12.00                 | 1.24                   | 3.66                   |
|                        | 9.67                  | 12.02                 | 1.24                   | 3.67                   |
|                        | 9.67                  | 12.07                 | 1.25                   | 3.74                   |
|                        | 9.67                  | 12.10                 | 1.25                   | 3.78                   |
|                        | 9.64                  | 12.05                 | 1.25                   | 3.74                   |
|                        | 9.67                  | 12.10                 | 1.25                   | 3.77                   |
|                        | 9.67                  | 12.10                 | 1.25                   | 3.78                   |
|                        | 9.57                  | 11.76                 | 1.23                   | 3.41                   |
|                        |                       |                       |                        | 0.33%                  |
|                        |                       |                       |                        | 0.38%                  |
|                        |                       |                       |                        | 0.24%                  |
|                        |                       |                       |                        | 1.21%                  |
| Column-to-column ($n=5$) | 9.24                  | 11.43                 | 1.24                   | 3.41                   |
|                        | 9.33                  | 11.62                 | 1.24                   | 3.56                   |
|                        | 9.52                  | 11.95                 | 1.26                   | 3.78                   |
|                        | 9.67                  | 12.10                 | 1.25                   | 3.78                   |
|                        | 9.38                  | 11.76                 | 1.25                   | 3.70                   |
| Day-to-day ($n=5$)     | 9.43                  | 11.71                 | 1.24                   | 3.56                   |
|                        | 9.38                  | 11.76                 | 1.25                   | 3.70                   |
|                        | 9.52                  | 11.95                 | 1.26                   | 3.78                   |
|                        | 9.67                  | 12.10                 | 1.25                   | 3.78                   |
|                        | 9.00                  | 11.43                 | 1.27                   | 3.77                   |
| Batch-to-batch ($n=5$) | 9.52                  | 12.05                 | 1.27                   | 3.93                   |
|                        | 9.05                  | 11.48                 | 1.27                   | 3.78                   |
|                        | 9.67                  | 11.86                 | 1.23                   | 3.41                   |
|                        |                       |                       |                        | 3.52%                  |
|                        |                       |                       |                        | 2.67%                  |
|                        |                       |                       |                        | 1.44%                  |
|                        |                       |                       |                        | 5.21%                  |

3.3. Stability and Reproducibility of the Chiral Monolithic Column

The stability and reproducibility of the chiral monolithic column were tested with benzoin as the chiral object, as shown in above table 1. Run-to-run referred to 10 consecutive injections of the same monolithic column. Day-to-day referred to the continuous measurement of the same monolithic
column for 5 days. Column-to-column referred to the reproducibility of five monolithic columns prepared in parallel from the same batch of solution. Batch-to-batch referred to the reproducibility of an overall column test of 5 batches of the same ratio. As can be seen from table 1, the prepared chiral monolithic column had good stability and reusability, and the method of preparing chiral monolithic column had good reproducibility.

The chiral separation test of benzoin was performed. After 100 consecutive injections, the resolution was tended to decrease, and the resolution was 3.25, further indicating that the chiral monolithic column had good repeatability.

4. Conclusion
A new type of HMC was prepared by sol-gel method in a stainless steel column, and then KH560 was connected to the surface through a dynamic cycle to form epoxy groups on the surface. PEI was grafted onto the monolithic column to form a large number of amino groups. After grafting glutaraldehyde onto the monolithic column, BSA was dynamically grafted onto the modified hybrid monolithic column using the Schiff-base method to successfully prepare a chiral monolithic column. The conditions for grafting BSA were optimized, and the maximum grafting amount was 56.1 mg/g. The chiral monolithic column had good stability and reusability.

Acknowledgments
This work was supported by the National Natural Science Foundation of China (No. 31501447), the Natural Science Foundation of Hebei Province (No. B2016201210) and Hebei University College Student' Student Innovation and Entrepreneurship Training Program (No. 2019187).

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