Preparation and Chiral Separation of a Novel Immobilized Cellulose-Based Chiral Stationary Phase in High-Performance Liquid Chromatography

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Introduction

Many drugs, natural products and food compounds are chiral, with their enantiomers often showing different or even opposing pharmacology, toxicity and metabolic activities (1). Currently, many top-selling drugs around the world are single enantiomers with the desired biological activity (2). Moreover, the attainment of pure enantiomers from racemates is an important concern in the stereochemical area. Among many other methodologies, high-performance liquid chromatography (HPLC) using chiral stationary phase (CSP) affords one of the most direct and effective approaches for enantiopurification.

During the past two decades, CSPs have advanced rapidly. The majority of polysaccharide-based CSPs employed have been cellulose-based columns (3). Polysaccharide derivatives that were coated on silica gel as CSPs appeared in the 1980s (4, 5). However, solvents that can swell or dissolve the derivatives could not be used as mobile phases, because the chiral selectors were physically coated onto the surface of the silica gel. Accordingly, coated CSPs were only amenable for use in a limited range of eluents, which were usually mixtures of non-polar solvents and alcohols used in normal phase mode (6, 7). To overcome the solubility of the coated selectors, Okamoto et al. first bonded the polysaccharide to a γ-aminopropyl silica gel matrix using a disiocyanate as a spacer that was expected to react with the free amino groups on the matrix surface and the hydroxyl groups of the polysaccharide (8). Because polysaccharide derivatives were covalently bonded onto silica gel, the CSP could be applied with a much broader range of solvents as mobile phases (9–17), which would enhance the success rate in enantioseparation.

As for amylose-based CSPs, Chiralpak IA is a most successful immobilized CSP using amylose 3,5-dimethylphenylcarbamate as its chiral selector (18). Thus far, many functional groups have been used to modify cellulose and have been further immobilized onto silica gel used as CSPs; for example, cellulose 3,5-dimethylphenylcarbamate (Chiralpak IB) (14), cellulose tris(3,5-dichlorophenylcarbamate) (Chiralpak IC) (19) and azido cellulose phenylcarbamate (20).

Cellulose tris(p-chlorophenylcarbamate) used as chiral selector that was coated onto silica gel was reported by Okamoto et al. (5). In this paper, cellulose p-chlorophenylcarbamate was first chemically immobilized onto silica gel by Staudinger reaction. The enantioseparation results showed that the CSP afforded high enantioseparation ability towards structurally diverse chiral compounds in either normal or reversed-phase mode.

Materials and Methods

Equipments

Nuclear magnetic resonance (NMR) was carried out on a Bruker ACF300FT-NMR spectrometer with tetramethylsilane as internal standard. Fourier transform infrared (FTIR) was performed on a Bio-Rad TFS156 instrument. Elemental analysis was determined on a PerkinElmer 2400CHN analyzer. The columns were packed using an Alltech pneumatic HPLC pump. Evaluation of the column was performed on an HPLC system, which comprised a Lib Alliance HPLC Series iii system, a Lib Alliance Model 201 ultraviolet-visible (UV-vis) detector and a 7725i injector equipped with a 20-μL sample loop.

Chemicals and reagents

Microcrystalline cellulose [degree of polymerization (DP) ≈ 200] and p-chlorophenyl isocyanate were purchased from Shanghai Hengxin Chemical Reagent Co. Amino propyltriethoxysilane and Compounds 9, 12-15, 20-22, 24, 29 and 30 were obtained from Alfa Aesar (Tianjin, China); Compounds 1–8 were provided by Professor Ding-Qiao Yang’s lab, and the other compounds were obtained from Professor Zhao-Yang Wang’s lab. HPLC-grade hexane, acetonitrile, isopropyl alcohol (IPA), methanol and ethanol were purchased from Tianjin Damao Chemical Reagent Co. Deionized and distilled water was used throughout the study. Silica gel (5 μm, 500 Å, 300 m²/g) was purchased from Fuji Silysia Chemical Ltd. (Aichi, Japan).

Preparation of mobile phases and samples

Triethylammonium acetate buffer (TEAA) was prepared by adding acetic acid to a solution containing 0.1% (v%) of
triethylamine to adjust to the desired pH 4.0. NaClO₄ aqueous solution was controlled at the concentration 0.30 mol / L. All buffers were filtered through a 0.45-μm membrane and degassed before use. The samples were prepared at a concentration of approximately 1 mg/mL. Ten microliters of the sample was injected. All chromatographic experiments were carried out at room temperature.

**Preparation of CSP**

Figure 1 shows the synthetic route to the CSP.

**Preparation of 6-azido-deoxy cellulose (Compound B)**

Microcrystalline cellulose was functionalized with a p-toluene sulfonyl chloride in N,N-dimethylacetamide/LiCl system to afford 6-tosyl-cellulose (Compound A) according to the reported method (21). FTIR (cm⁻¹, KBr): 3524 (OH), 3066 (C-Hαrom), 1598, 1496, 1455 (C-O), 1367, 1176 (-SO₂-), 1060 (C-O-C). Elemental analysis, found: C 49.85%, H 4.97%, S 1.88%, N 2.18%.

**Preparation of amino functionalized silica gel**

Silica gel (20 g, dried at 180 °C under 0.1 mm Hg for 4 h) was suspended in a mixture of 250 mL toluene in nitrogen atmosphere. Five millilitres of 3-aminopropyltriethoxysilane was added to the reaction flask. The mixture was refluxed at 110 °C for 15 h. The resultant product was washed by soxhlet extraction with acetone for 24 h (22). Elemental analysis: C 5.21%, H 4.68% and N 13.83%.

**Chemical immobilization of 6-azido-2, 3-di(p-chlorophenyl carbamoylated) cellulose onto amino functionalized silica gel**

Amino functionalized silica gel (4.00 g) was stirred in anhydrous tetrahydrofuran (THF) (30 mL) through which a continuous carbon dioxide was bubbled. After 20 min, a solution of 6-azido-2, 3-di(p-chlorophenyl carbamoylated) cellulose (8.40 g) in anhydrous THF (30 mL) was added. Stirring was continued for another 5 min, then triphenylphosphine (2.00 g) in 25 mL THF was added. The mixture was stirred for 24 h with bubbling carbon dioxide at room temperature, and then filtrated. Excess non-bonded Compound C was washed with acetone overnight after the procedure (iii). The CSP was obtained, marked as CSP-1.

**Evaluation of the column**

Using the standard slurry method, the CSP was packed into a stainless steel column (250 × 4.6 mm i.d.) at a pressure of 8,000 psi. CCl₄-dioxane (2:1, v/v) was used as slurry solvent and methanol was used as packing solvent.
The columns packed with CSP-1 afforded efficiency of Approximately $3.8 \times 10^4$ plates/m using biphenyl as test probe in normal phase mode [hexane-IPA (90:10, v/v)].

**Calculations**

The following resolution parameters were determined: $k$ was calculated from the equation $k = (t_R - t_0)/t_0$, where $t_R$ referred to the retention time and $t_0$ was the dead time. The $t_0$ for the analytical column was measured from the retention of sodium nitrate in reversed phase mode and 1,3,5-tri-tert-butylbenzene in normal phase mode. The void volume was 1.60 mL. The separation factors ($\alpha$) were calculated using $\alpha = k_2/k_1$, where $k_1$ and $k_2$ were the retention factors for the first and second eluted enantiomers, respectively. The resolution ($R_s$) was calculated from the equation $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where $t_1$ and $t_2$ were the retention times of the first and the second enantiomers, respectively, and $w_1$ and $w_2$ were the peak widths (based on USP standards).

**Results and Discussion**

Chiral separations were conducted in normal and reversed-phase mode. Structures of chiral compounds mentioned in this work are shown in Figure 2.

**Enantiomeric separation in normal phase mode**

Mixtures of hexane and alcohols were used in the enantioseparation. Fourteen analytes were effectively separated, with $k_1$ calculated from the equation $Rs = 2(t_2 - t_1)/(w_1 + w_2)$, where $t_1$ and $t_2$ were the retention times of the first and the second enantiomers, respectively, and $w_1$ and $w_2$ were the peak widths (based on USP standards).
ranging from 1.04 to 4.13 and $Rs$ ranging from 0.93 to 12.26. Mobile phase, retention factor ($k$), separation factor ($\alpha$) and resolution ($Rs$) of all compounds separated in normal phase mode are shown in Table I.

The mechanism of enantioselectivity of polysaccharide derivatives has been studied by several research groups. Fukui et al. concluded that $\pi-\pi$ interaction between the solute and CSPs played an important role in the chiral recognition process (24, 25). O’Brien reported that the difference in H-bonding of the two enantiomers with the polysaccharide derivatives was another factor that contributed to the enantioselectivity (26).

Accordingly, $\pi-\pi$ interaction and H-bonding might be the intrinsical forces for chiral resolution of the polysaccharide in normal phase mode. Because there is no phenyl in the structure of Compound 9, H-bonding between $S=O, -NH_2$ of the analyte and N-H, C=O of CSP-1 might be the dominant factor contributing to its $Rs$ to 2.54.

Chromatograms of several compounds separated on the CSP-1 in normal phase mode are presented in Figure 3.

### Table I

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*Separation conditions: flow rate, 1.0 mL/min; UV detector, $\lambda = 254$ nm; Compound 9, $\lambda = 228.$ nm.

**Enantiomeric separation in reversed-phase mode**

Methanol, ethanol, acetonitrile and IPA mixed with an appropriate proportion of H₂O or TEA or NaClO₄ solution were used as mobile phases. Enantioseparation data of 16 solutes separated successfully in reversed phase mode are listed in Table II.

The helical backbone and side chain of the cellulose derivative keep the analyte in the chiral cavities environment, so it could be deduced that solutes would be wrapped into the heliced polymer cavity. In addition, the side chains were exposed to the outside, which would help to form $\pi-\pi$ interaction with solutes (27).

Compound 19 is a racemate that possesses no aromatic moiety in its structure, hence there is no sorbent-solute $\pi-\pi$ interaction in its chiral separation process. However, it afforded $Rs$ up to 1.66 in reversed-phase mode. This may be due to H-bonding and steric effects. In contrast to Compound 14 separated in normal phase mode, Compound 20 was separated in reversed-phase. Compound 14 contained additional phenyl rings while Compound 20 embodied two free phenolic groups.

![Chromatogram A](image1)

![Chromatogram B](image2)

![Chromatogram C](image3)

![Chromatogram D](image4)

**Figure 3.** Chromatograms for some chiral compounds separated on the CSP-1 in normal phase mode (separation conditions and results are shown in Table I): Compound 9 (A); Compound 28 (B); Compound 29 (C); Compound 30 (D).
As for Compound 14, the additional phenyl groups might afford additional π–π interaction sites and steric hindrance. The two free phenyl hydroxyl groups of Compound 20 made H-bonding interaction with mobile phase and CSP-1 possible.

Chromatograms of several representative compounds separated on the CSP-1 in reversed phase mode are presented in Figure 4.

Reproducibility of chiral stationary phase
To investigate the reproducibility of the CSP, several batches of the CSP have been synthesized. Standard sample Compound 3 was separated on different batches of CSP in the same condition. Three batches (marked as CSP-1, CSP-2 and CSP-3, respectively) were chosen as representatives. The results are listed in Table III. Chromatograms are shown in Figure 5.

Stability of the chiral stationary phase
The stability of the immobilized CSP would undoubtedly be a determining factor for the repeatability of separation methods and the column lifetime. Six hundred injections were carried out in both normal and reversed-phase modes within two months. Compound 1 was repetitively injected on the CSP-1 with chromatographic data listed in Table IV and chromatograms presented in Figure 6.

Owning to its immobilized nature, non-standard solvents were tried to be used as eluents. Surprisingly, total separation of Compound 28 (Rs = 12.26) was achieved using hexane-CHCl₃-ethanol (68:30:2, v/v/v) which is shown in Figure 3B. Additionally, the last process of the preparation of the CSP is to wash the CSP with acetone overnight by soxhlet extraction, aiming at washing off the non-bonded 6-azido-2,3-di(p-chlorophenylcarbamoylated) cellulose. Consequently, the CSP is resistant to non-standard solvents such as acetone, CHCl₃.

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*Separation conditions: flow rate, 0.5 mL/min; UV detector, λ = 254 nm; Compound 19, λ = 228 nm.

Figure 4. Chromatograms for some chiral compounds separated on the CSP-1 in reversed phase mode (separation conditions and results are shown in Table II): Compound 6 (A); Compound 21 (B); Compound 22 (C); Compound 23 (D).
Conclusion
In summary, a novel chemically immobilized cellulose-based CSP has been prepared using Staudinger reaction between 6-azido-2,3-di(p-chlorophenylcarbamoylated) cellulose and amino functionalized silica gel. Chiral compounds were effectively enantioseparated in either normal phase mode or reversed-phase mode. After analysis of the enantioseparation data and structures of the chiral compounds, synergistic forces comprising inclusion, π-π interaction, H-bonding and steric effects may contribute to enantioseparation of racemates. Additionally, results revealed good reproducibility and stability of the CSP.

Acknowledgments
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