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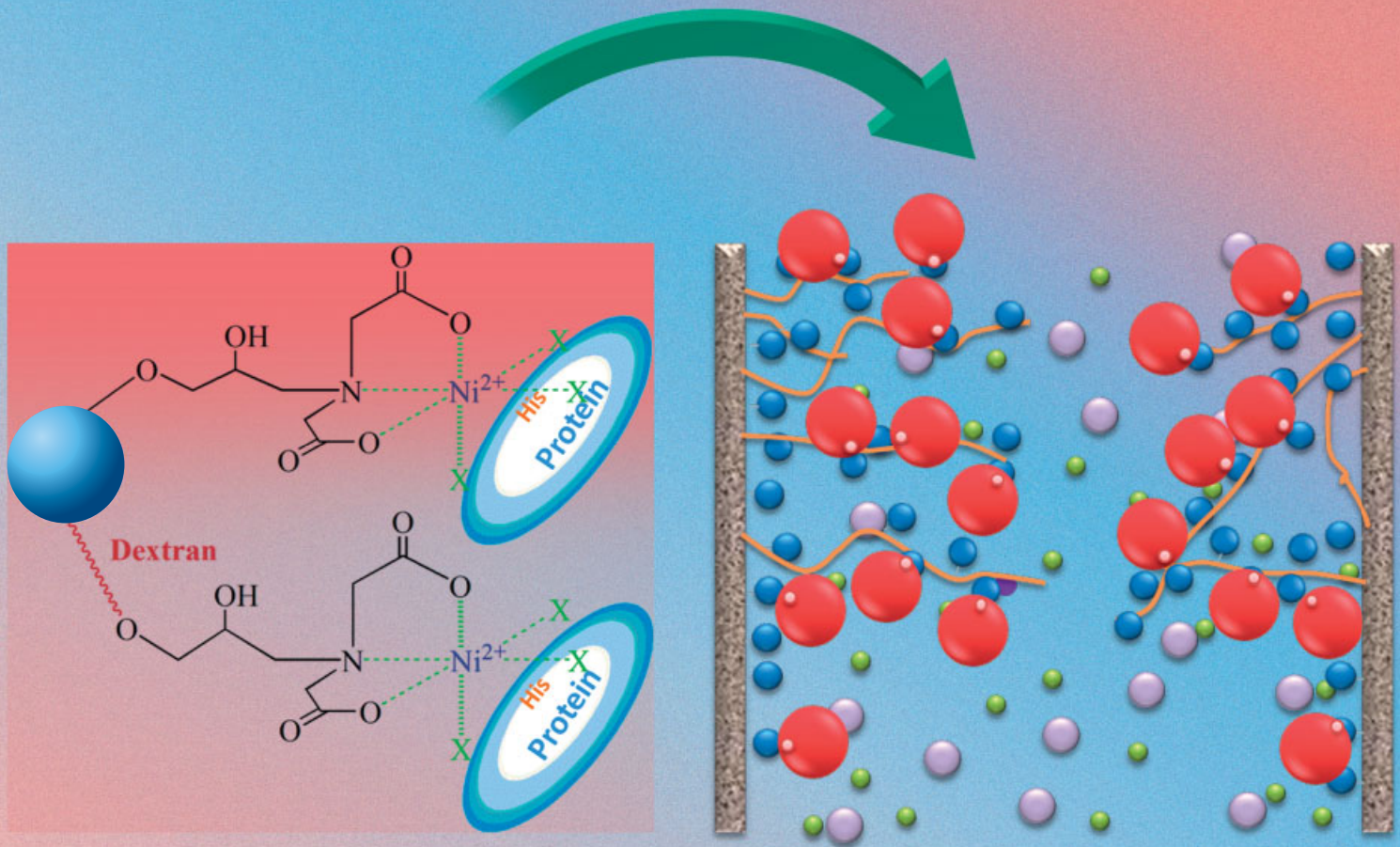

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Research Article

Preparation of cyclodextrin-modified monolithic hybrid columns for the fast enantioseparation of hydroxy acids in capillary liquid chromatography

Cyclodextrins and their derivatives are one of the most common and successful chiral selectors. However, there have been few publications about the use of cyclodextrin-modified monoliths. In this study, organic hybrid monoliths were prepared by the immobilization of derivatized β-cyclodextrin alone or with L-2-allylglycine hydrochloride to the polyhedral oligomeric silsesquioxane methacryl substituted monolith. The main topic of this study is a combined system with dual chiral selectors (L-2-allylglycine hydrochloride and β-cyclodextrin) as monolithic chiral stationary phase. The effect of L-2-allylglycine hydrochloride concentration on enantioseparation was investigated. The enantioseparation of the four acidic compounds with resolutions up to 2.87 was achieved within 2.5 min on the prepared chiral monolithic column in capillary liquid chromatography. Moreover, the possible mechanism of enantioseparation was discussed.

Keywords: Capillary liquid chromatography / Cyclodextrins / Enantioseparation
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1 Introduction

The development of chiral separation methods is an important task for the production of enantiopure chemicals and pharmaceuticals [1–4]. A great variety of analytical methods are based on chromatographic techniques, such as HPLC, capillary liquid chromatography (cLC), GC, SFC and electromigration techniques CE and CEC [5–8]. The use of a monolithic stationary phase in cLC or CEC displays many advantages in enantioseparations such as high separation efficiency, low solvent consumption and low sample requirement [9–13]. Moreover, organic polymer monoliths, mainly polyhedral oligomeric silsesquioxane (POSS)-based monoliths, possess excellent mechanical stability [14]. Their unique inorganic–organic hybrid nanostructures, facile chemical modification and oxidation resistance properties make POSSs an ideal material for preparation of hybrid monoliths [15–17].

Organic polymer monoliths column could be prepared by various methods such as radical polymerization (thermally or photoinduced) [18], polycondensation reaction [19] and ring-opening metathesis polymerization [20] depending on the type of stationary phase. In our recent studies, we have introduced POSS-methacryl substituted (POSS-MA) into monolithic column by free radical polymerization [21, 22]. Radicals are formed from the initiator, which initiates the polymerization of POSS monomers. Continued polymerization increases the nuclei size, causing coalescence to form clusters, which join to form a homogeneous structure [23–25]. Cyclodextrins (CDs) and their derivatives have been one of the most common and successful chiral selectors [26, 27]. However, there has been relatively less published work on the use of CDs as monolithic monomers [28–32]. The possible reasons may be that the ability of CDs as monolithic monomer to resolve enantiomers is poor. For that reason attempts have been undertaken to enhance the resolution (Rs) [33].

We proposed a combined system with dual chiral selectors as monolithic chiral stationary phase (CSP). To the best of our knowledge, this combined system as a monolithic CSP has not been investigated yet. However, many papers dealing with the use of dual chiral CSPs are well known in GC,

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Abbreviations: **AGH**, L-2-allylglycine hydrochloride; **CDs**, cyclodextrins; **cLC**, capillary liquid chromatography; **CSP**, chiral stationary phase; **NP**, normal phase; **POSS-MA**, polyhedral oligomeric silsesquioxane methacryl substituted monolith; **Rs**, resolution

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CE and also as mobile phase additives in HPLC [34, 35]. In our case, the derivative of β-CD in combination with L-2-allylglycine hydrochloride (AGH) serves as monolithic CSP. As a result, a significant enhancement of enantioseparation of hydroxy acids may be observed under appropriate conditions. Moreover, the possible explanation of mechanism of enantioseparation was proposed.

2 Materials and methods

2.1 Chemicals and materials

POSS-MA (cage mixture, n = 8, 10, 12) was purchased from Aldrich (Milwaukee, WI, USA). 1-Propanol and PEG Mn 400 (PEG 400), benzene and its derivatives, 2-hydroxy-2-(4-hydroxyphenyl)propanoic acid, DL-4-hydroxy-3-methoxymandelic acid, 4-trifluoromethyl mandelic acid, 2-hydroxy-2-phenylpropanoic acid, 10-undecenoyl chloride, phenyl isocyanate and γ-methacryloxypropyltrimethoxysilane were purchased from Sigma (St Louis, MO, USA). DL-Mandelic acid, 2-hydroxy-2-(2-hydroxyphenyl)propanoic acid and 3-amino-3-phenylpropanoic acid were obtained from J & K Scientific (Pekin, China). Phenylthiohydantoin DL-glutamic acid was supplied by Weibo Chemical (Guangzhou, China). Azobisisobutyronitrile (AIBN) and β-CD were purchased from Shanghai Chemical Plant (Shanghai, China). AGH was bought from TCI (Tokyo, Japan). A fused-silica capillary with 75 μm id and 365 μm od was purchased from Reafine Chromatography (Hebei, China). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Tianjin Fuchen Chemical Reagents Factory (Tianjin, China).

2.2 Instrumentation

The experiments were carried out on an LC system equipped with an Agilent 1100 (Hewlett–Packard) micropump, a UV detector (K-2501, Knauer, Germany) and a 7725 injector with a 20 μL sample loop. A T-union connector served as a splitter with one end connected to the capillary monolithic column and the other end to a blank capillary (95 cm long, 50 μm id and 365 μm od). The data were collected at 214 or 254 nm, and processed by a chromatography workstation (Beijing Cailu Scientific Instrument, Beijing, China). The split ratio was controlled at about 1:400 for the 50–200 μm id capillary columns. The outlet of the monolithic column was connected to an empty fused-silica capillary (50 μm id and 365 μm od) with a Teflon tube, where a detection window was made by removing a 2 mm length of the polyimide coating in a position of 5.0 cm from the separation column outlet. Chromatographic measurements were made at room temperature. The dead time of the column was determined by injecting 1 μL samples of KNO_3 (0.01 mg/mL).The permeability of columns (P) was determined by using Darcy's law: $P = F\eta L/(\pi r^2 \Delta P)$ [36], where η is the mobile phase viscosity, L and r (m) are effective length and inner radius of the column, F (m^3/s) is the flow rate of mobile phase and ΔP is the pressure drop across the column. The permeability was measured by using 40:60 v/v ACN/water. The viscosity of ACN/water (40:60 v/v) was obtained from ref. [37].

2.3 Synthesis of CD derivative

The CD derivative was synthesized according to the recently reported method [38]. In brief, β-CD (3.0 g) was dissolved in the solution of 50.0 mL anhydrous pyridine, and then 10-undecenoyl chloride (3.279 mL) was dropped slowly. The reaction mixture was stirred at 100°C for 24 h. Then, phenyl isocyanate (12.0 mL) was added, and the resulting mixture was stirred at 100°C for 10 h. After removal of pyridine and the unreacted phenyl isocyanate under reduced pressure, product was isolated by silica gel column chromatography with 1/4 v/v mixture of petroleum ether and ethyl acetate. The product was characterized by MALDI-TOF MS (m/z): 3704 was molecular weight of [mono(10-undecenoyl)-perphenylaminocarbonyl β-CD+Na^+]. Characterization data for 2-mono(10-undecenoyl)-perphenylaminocarbonyl β-CD: IR (cm^{-1}, KBr, Supporting Information Fig. S1: 3397 and 3312 (N–H), 3056 (CH=CH–H), 2938, and 2850 (C–H), 1732 (C=O), 1604, 1539, 1500, and 1447 (arom C=C), 1318 (C–N), 1228 (O=C–O), 1086, 1057, and 1029 (C–O–C), 754, and 692 (Ar-H).

2.4 Preparation of pristine monolithic columns

The capillary was washed by 0.1 M NaOH, water, 0.1 M HCl and water [39]. A solution of 50% v/v γ-methacryloxypropyltrimethoxysilane dissolved in methanol was prepared. The capillary was then washed with 15-column-volume of the solution. The capillary was immersed into a water bath at 60°C for 12 h with both ends sealed by rubbers. Finally, the capillary was washed with methanol and dried by a nitrogen stream.

The mixture of POSS-MA, propanol, PEG 400 was prepared with AIBN. The homogeneous mixtures were purged with nitrogen for 15 min. After that, the mixture was introduced into a modified capillary to a length of 30 cm. Then with both ends sealed, the capillary was heated at 60°C by water bath for 12 h. After the polymerization, the column was washed by methanol.

2.5 Immobilization of CD derivative

The monolithic column was filled with solution of CD (0.25 g/mL) or CD with AGH (3.0 or 5.0 mg/mL) in methanol. The capillary was heated at 70°C by water bath for 12 h to bond CD derivative to POSS-MA. Afterwards, the column was washed by methanol.

2.6 IR and SEM

The polymerization mixture in the centrifuge tube was polymerized under the same conditions as the preparation of

Table 1. Effect of synthesis parameters on the formation of hybrid POSS-MA monoliths[a)]

Column	Propanol[b)] (*v/v* %)	PEG 400[b] (*v/v* %)	Permeability[c)] ($\times 10^{-14}$ m^2)	Optical microscope images
A	67.74	32.26	Too hard to pump	
B	65.63	34.38	7.56	
C	64.52	35.48	31.61	
D	66.88	33.12	4.15	

a) Other preparation conditions: polymerization temperature 60°C, POSS-MA 45 mg, AIBN 2 mg.
b) The total volume of propanol/PEG400 mixture was 160 μL.
c) Permeability, $P = F\eta L/(\pi r^2 \Delta P)$, *F*-linear velocity of mobile phase; η-dynamic viscosity of mobile phase; *L*-effective column length; *r*-inner radius of column; Δ*P*- pressure drop across the column [28], measured under room temperature.

monolithic capillary columns. The synthesized matrix was cut into small pieces. Then the pieces were dipped into ethanol, and ultrasonicated for 30 min. The washing step was repeated for 3 times. The matrix was dried under vacuum at 60°C for 24 h. The obtained matrix was used for FTIR. FTIR spectra were measured on Thermo Nicolet 380 spectrometer with KBr pellets (Nicolet, Wisconsin, USA). KBr pellets, containing around 1 mg monolith sample and 100 mg KBr, were prepared by powder compressing machine.

After thoroughly flushing out residues, the monoliths were laid aside for several days at room temperature to allow the methanol evaporate from the pore structure. Then they were cut into 3–4 mm length pieces, and sputtered with gold with an accelerating voltage of 20 kV for 4 min. SEM images were obtained using the secondary electron imaging mode of a JEOL JSM-5600 scanning electron microscope (JEOL, Tokyo, Japan).

3 Results and discussion

3.1 Preparation and evaluation of POSS–MA hybrid monolith

The preparation condition of the monolithic column has been investigated. Several kinds of porogenic systems, such as propanol/1,4-butanediol/PEG 10000, propanol/1,4-butanediol and toluene/dodecanol, were examined. Finally, the mixture of propanol/PEG 400 was chosen due to its favorable solubility. The amounts of PEG 400 and propanol used for the preparation of monolithic column were listed in Table 1. The permeability of monolithic materials was measured. It was found that the permeability was increased as the volume percentage of propanol in the porogenic mixture (propanol/PEG 400) was decreased from 67.74 to 64.52% (as columns A and C). When the volume percentage of PEG 400 was 32.26% (column A), it was too dense to pump through the obtained monolith. However, it was too loose when the volume percentage of PEG 400 was 35.48% (column C). Only when the volume percentage of PEG 400 was 33.12% (column D), a homogeneous monolith with the permeability of 4.15×10^{-14} m^2 was obtained.

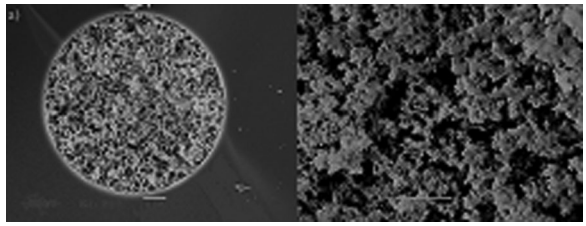

Figure 1. SEM of the monolithic column with magnifications of (a) 1000 and (b) 5000.

The morphology of monolithic column POSS-MA was characterized by SEM, and the result was given in Fig. 1. It can be easily observed that the monolithic material was successfully formed inside the capillaries, and tightly anchored to the inner wall of the capillary without any disconnection. Additionally, some macropores with several micrometers could be observed.

Figure 2 shows the IR of bare monolithic material and CSPs immobilized chiral selectors. After immobilization of chiral selectors appearance characteristic absorptions of derivative CD (N–H stretch at 3423 cm^{-1}, C–H stretch at 2973 cm^{-1}, C=O stretch at 1728 cm^{-1}, C=C stretch in the aromatic ring at 1549 cm^{-1}) and AGH (C–N stretch at 1049 cm^{-1}, N–H wag at 847 cm^{-1}). The result confirms successful immobilization of chiral selectors.

The mechanical stability of the monolithic POSS-based column was investigated. The backpressure increased linearly ($R = 0.998$) as the flow rate increased (Supporting Information Fig. S2). These results indicated that the monolithic column possessed good mechanical stability. The reproducibility of the POSS-MA hybrid monolithic column was also investigated. The reproducibility was evaluated through the RSD for the retention factor of thiourea. The run-to-run ($n = 5$) reproducibility was 0.53%. Six monolithic columns were prepared with the same conditions, three from one batch and three from different batches. The RSD values for the column-to-column ($n = 3$) and batch-to-batch ($n = 3$) were 5.17 and

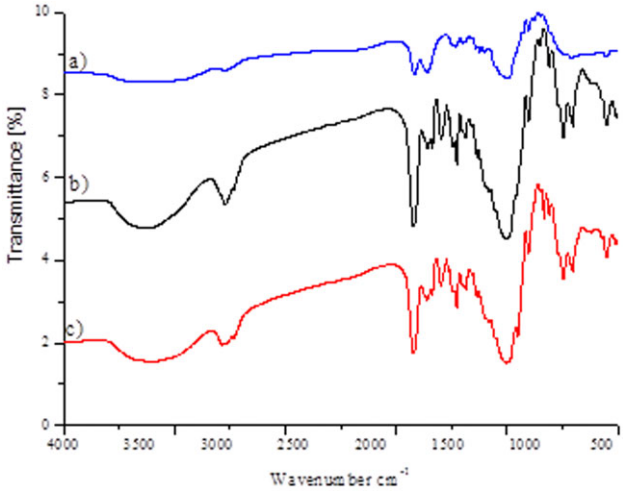

Figure 2. IR spectra of the bare monolith and chiral stationary phase. (a) Bare column; (b) with derivative CD; (c) with derivative CD and AGH.

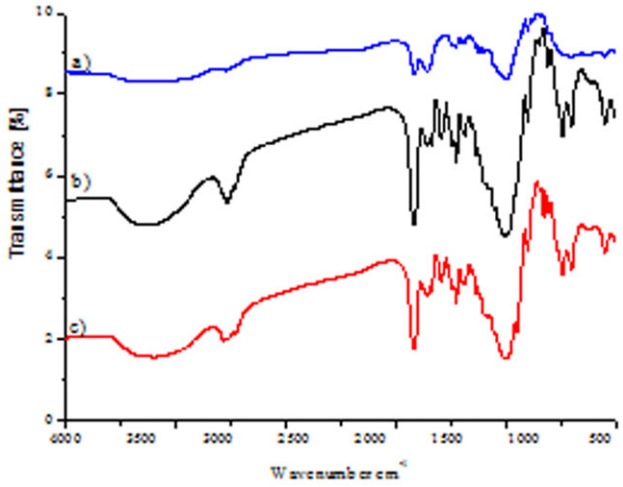

Figure 3. Separation of alkylbenzenes on POSS-MA hybrid monolithic column. Experimental conditions for cLC: mobile phase, ACN/H_2O 40:60 v/v; column length, 25 cm; detection wavelength, 215 nm; injection volume, 2 μL. Analytes: (1) thiourea, (2) benzene, (3) toluene, (4) ethylbenzene, (5) propylbenzene, (6) butylbenzene.

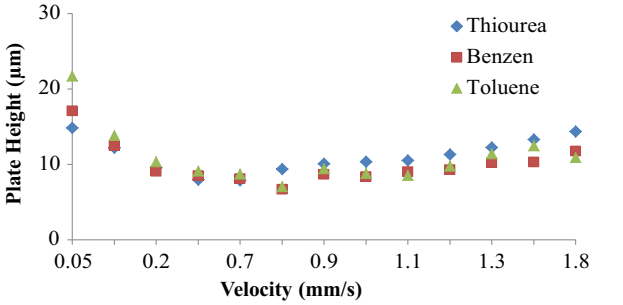

Figure 4. Relationship of the plate height on the velocity of mobile phase on the POSS-MA hybrid monolithic column.

6.42%. The long-term stability of the monolithic columns was observed with no significant changes in the retention time over 1000 runs. The RSD still remained and did not exceed 3%.

The study of chromatographic performance of the POSS-MA hybrid monolithic column was performed. Figure 3 shows the Rs values in the range of $2 < Rs < 6$ of alkylbenzenes with good peak shapes on the POSS-MA hybrid monolithic column.

Figure 4 presents the relationship between the velocity and the plate height for thiourea, benzene and toluene. No significant changes in column efficiency were observed with linear velocities ranging from 0.4 to 1.2 mm/s.

3.2 Enantioseparations on CD and CD/AGH hybrid monolithic columns

The raw POSS-MA hybrid monolith was chemically modified with CD or CD and AGH for chiral separation. The CD and CD/AGH chemically modified hybrid monoliths were characterized by SEM (Supporting Information Fig. S3), and no obvious difference in the cross-section and in permeability of the hybrid monoliths before and after chemical modification could be found.

3.2.1 Enantioseparation in the reversed-phase (RP) mode

The series of CSPs were prepared by the chemical immobilization of various kinds of chiral compounds such as AGH, CD with AGH, CD to the POSS-MA monolith. Enatioseparation was not observed on the AGH monolithic column. Satisfactory enantioseparation was obtained only for monolithic CSP serve as the derivative of β-CD in combination with AGH. The influence of AGH addition on the enantioseparation of acidic compounds was studied at constant (0.25 g/mL) concentration of β-CD. Figure 5 and Table 2 show that the CD/AGH monolithic column possessed higher chiral recognition ability than CD monolithic column. Four hydroxy acids exhibited excellent enantioseparations in the retention time less than 2.5 min when the concentration of AGH exceeds 3.0 mg/mL. However, for 5.0 mg/mL concentration of AGH, the enantioselectivity decreased for all model compounds. The differences in enantioseparation capability of acidic compounds under two mobile phase with pH 4 and 8 was also investigated. The column CD-AGH at the pH 8 lost its enantioseparation ability.

This experiment showed that the addition of AGH to the CD polymerization mixture can lead to significant improvement in the enantioseparation of hydroxy acids. In this study AGH plays key role in enantioseparation. Hydroxy acids and AGH formed hydrogen bonds. For the six compounds, such as mandelic acid, chiral center is directly connected to the hydroxy group, which form hydrogen bond with AGH. This hydrogen bond can be considered to be one of the preferential interaction responsible for chiral recognition.

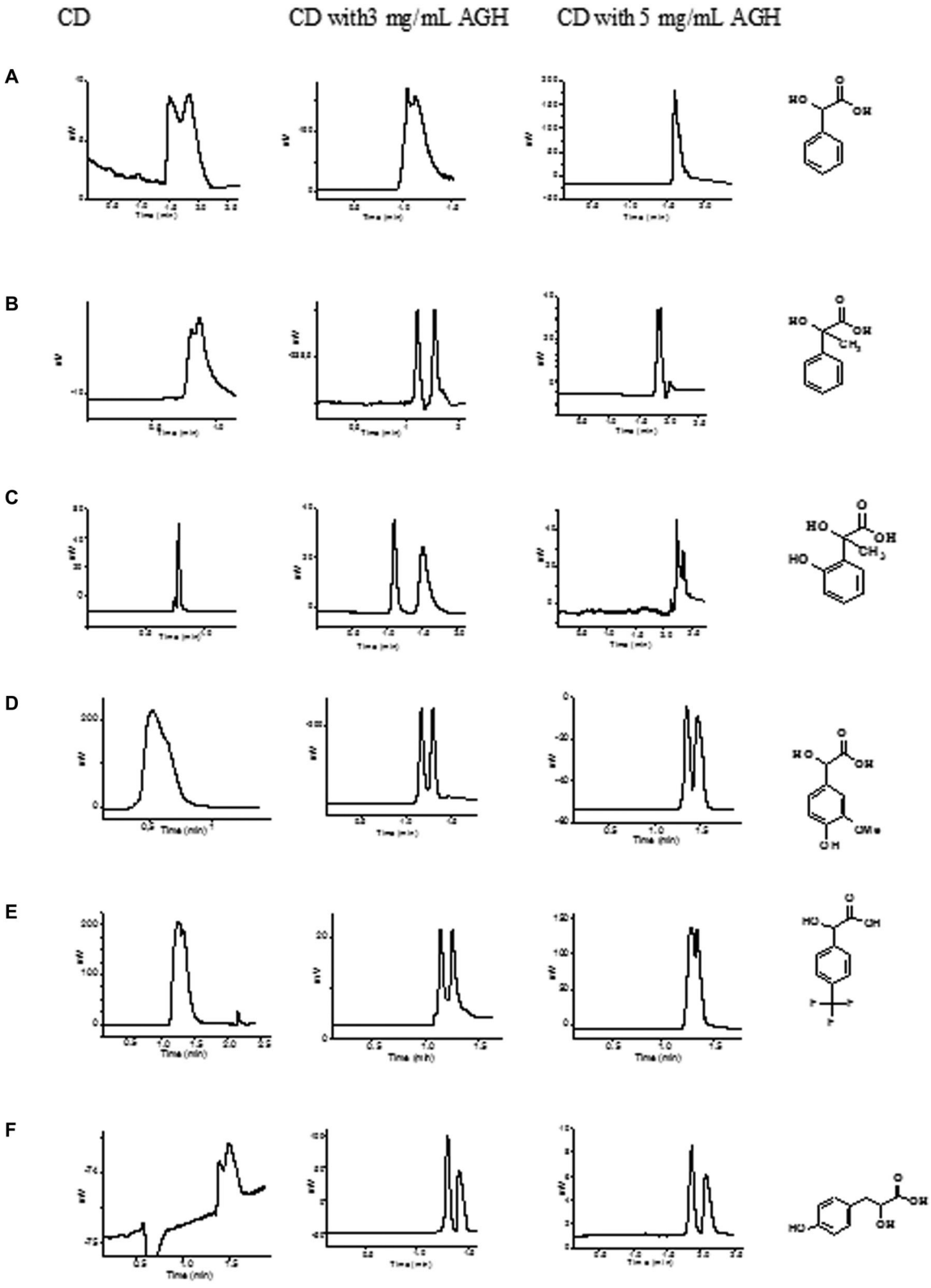

Figure 5. Chromatograms of enantioseparation of hydroxyacids on the CD and CD with 3.0 or 5.0 mg/mL AGH monolithic columns. Experimental conditions: mobile phase, 10 mM CH_3COONH_4 containing 40% v/v methanol at pH 4.3; column length, 25 cm; detection wavelength, 215 nm. Analytes: (a) DL-mandelic acid, (b) 2-hydroxy-2-phenylpropanoic acid, (c) 2-hydroxy-2-(2-hydroxyphenyl) propanoic acid, (d) DL-4-hydroxy-3-methoxymandelic acid, (e) 4-trfluoromethyl mandelic acid, (f) 2-hydroxy-2-(4-hydroxyphenyl)propanoic acid, (g) 3-amino-3-phenylpropanoic acid and (h) phenylthiohydantoin DL-glutamic acid.

Table 2. Enantioseparation results on the CD and CD/AGH hybrid monolithic columns

	CD pH = 4.0			CD/AGH pH = 4.0			CD/AGH pH = 8.0	
Comp.	k_1	k_2	α	k_1	k_2	A	Rs	k_1
a	0.24	0.26	1.08	0.21	0.34	1.62	0.82	0.19
B	0.12	0.15	1.25	0.14	0.43	3.07	2.05	0.11
C	0.35	–	–	0.50	1.50	3.00	2.87	0.21
D	0.20	–	–	0.25	0.49	1.96	1.46	0.18
e	0.06	0.08	1.39	0.13	0.29	2.23	1.06	0.09
f	0.16	0.19	1.19	0.28	0.52	1.89	1.49	0.18
g	0.21	–	–	0.23	0.28	1.22	0.64	0.21
h	0.27	0.32	1.17	1.87	2.33	1.24	0.51	0.29

a) Experimental conditions: mobile phases, 10 mM NH_4COOH containing 40% MeOH at pH 8.0 or 10 mM CH_3COONH_4 containing 40% MeOH at pH 4.0; column length, 25 cm; detection wavelength, 215 nm.

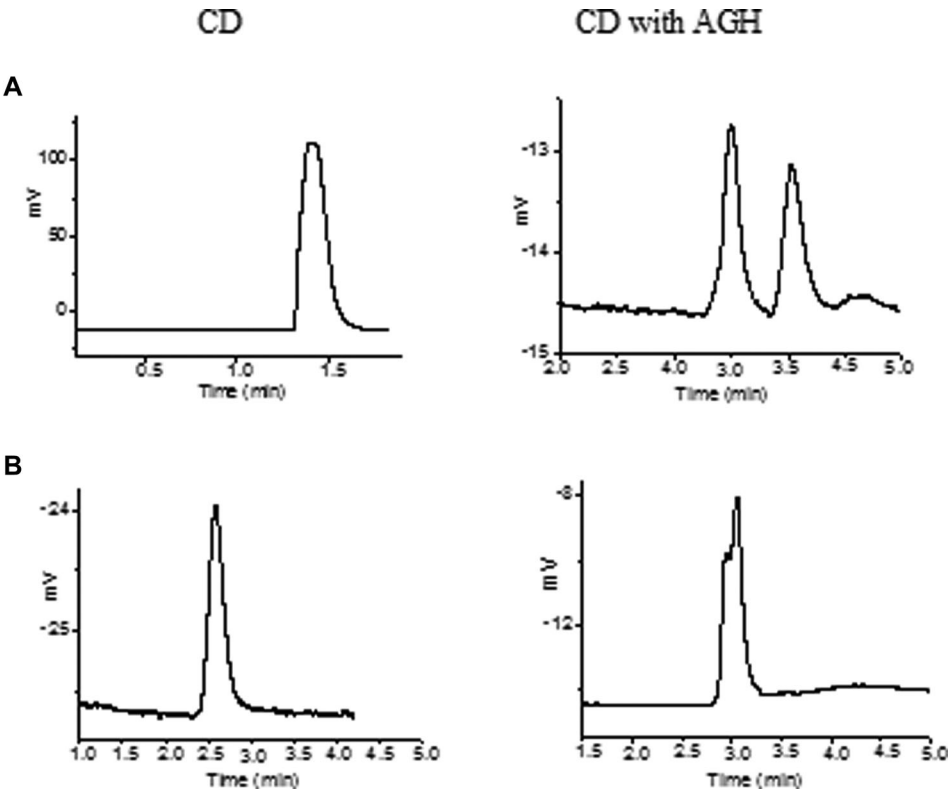

Figure 6. Chromatograms of enantioseparation of hydroxy acids on the CD and CD with 3 mg/mL AGH monolithic columns. Experimental conditions: mobile phase, *n*-hexane/propanol (80:20, v/v); column length, 25 cm; detection wavelength, 215 nm. Analytes: (a) 4-chloromandelic acid, (b) 2-hydroxy-2-phenylpropanoic acid.

Compared with 2-hydroxy-2-phenylpropanoic acid (compound b) and 2-hydroxy-2-(2-hydroxyphenyl) propanoic acid (compound c), enantioseparation of mandelic acid (compound a) showed poorer Rs, the weaker hydrogen interaction of the AGH with this compound apparently resulting in less chiral discrimination effect. The relative importance of hydrogen bonding can well be dependent on the steric hindrance and the structure of the molecule [40]. Compounds b and c have higher steric hindrance, which exhibited better enantioseparation ability. Taking DL-4-hydroxy-3-methoxymandelic acid (compound d), 4-trfluoromethyl mandelic acid (compound e), 2-hydroxy-2-(4-hydroxyphenyl)propanoic acid (compound f) for example, additional group –OH or $–CF_3$ bonding with the aromatic ring can provide additional sites for hydrogen bonding, which enhanced the enantioseparation, in comparison with compound a. 3-Amino-3-phenylpropanoic acid (compound g) and phenylthiohydantoin DL-glutamic acid (compound h) are different in the structure from other examined compounds. For these compounds, the chiral center is connected to the amino group, which can be the reason for the change in enatioseparation. The hydrogen bonding interaction around the chiral center of these compounds with the AGH is weaker than hydroxy acids with AGH. It

seems that the hydrogen bonding interaction, only intensified the retention but did not significantly improve the enantioseparation.

According to the "three-point" attractive interactions model [41], another interaction can take place such as electrostatic interaction, π–π interaction and dipole–dipole interaction.

The pI of AGH was determined using a graphical approach (Supporting Information Fig. S4). The equivalence point occurred at the pH = 6.22. If the amino acid is in a solution where the pH is above the pI, than both ends will be in deprotonated form. AGH at pH 8 possess a negative charge and provided repulsion to anionic hydroxy acids. It is well proofed that the enantioseparation depends directly on the affinity of the enantiomers to the chiral selectors [42]. The main mechanism for enatioseparation should be hydrogen bonding chiral compounds with AGH. This interaction can provide better enantioselectivity ability of CD through formation more stable complex hydroxy acids-CD in the presence of AGH compared to complex hydroxy acids-CD without addition AGH. For chiral recognition of CDs, the chiral center of the compounds, that form an inclusion complex must be near the rim of CD cavity, to generate strong contact with the cavity, such as a hydrogen bond [43].There is limited knowledge about the molecular interactions between AGH and β-CD and hydroxy acids. However, it can be explained that acids can form ternary complexes with amino acids and CDs.

3.2.2 Enantioseparation in the normal-phase (NP) mode

Chromatographic separation was also performed under NP mode. The mechanism of enantioseparation under NP mode is different from the RP mode. The inclusion complex formation does not contribute to chiral recognition in NP mode, because *n*-hexane is incorporated in the CD cavity. However, π–π interactions, dipole stacking and hydrogen bonding interactions are important for chiral recognition. The 4-chloromandelic acid was not separated under the RP mode, however it was resolved under the NP mode (Fig. 6). The presence of a 4-chloro substituent contributes to the enantioseparation, though the stronger π–π interactions between hydroxy acid and CD occurred due to the removal of electron density from the benzene ring, in comparison with compounds c, d and f with –OH groups, which increase the electronic density of the benzene ring, making weaker π–π interactions. When there is a lack of such π–π interaction, a chiral recognition cannot be achieved because the "three-point rule" requirement is not fulfilled. Significant improvement in enantioseparation can be still observed by addition AGH to CD polymerization mixture. This improvement is obtained through the hydrogen bonding interaction between acidic compounds and AGH.

4 Concluding remarks

The main of this study was to obtain a novel CD monolith with chiral recognition ability. The raw POSS-MA hybrid monolith was chemically modified with either single chiral selector β-CD or dual selectors β-CD/AGH with different content of AGH. AGH monolith column had no chiral recognition ability. The enantioseparations of chiral compounds on CD and CD/AGH monolithic columns were compared. The CD/AGH chiral monolithic column provides improvement in enantioseparation for specific group of compounds such as hydroxy acids. The enantioselectivity of the CD/AGH chiral monolithic column was evaluated in the NP and RP modes. Four hydroxy acids exhibited excellent enantioseparations in retention time less than 2.5 min. The main mechanism for enatioseparation should be hydrogen bonding interaction between acidic compounds and AGH. The hydrogen bonds reinforce the affinity of enantiomers to the cavity of CD, resulting in significant improvement in enantioseparation of hydroxy acids.

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The authors have declared no conflict of interest.

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