Chondroitin Sulfate Modified Stationary Phase with Mixed Mode of Hydrophilic Interaction and Strong Cation-Exchange for Capillary Electrochromatography

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Abstract
A novel column packing material, chondroitin sulfate-modified silica gel (ChS-silica), was developed for use as a stationary phase or capillary electrochromatography. The sulfonic acid groups on the ChS-silica supported electroosmotic flow at low pH and served as a strong cation-exchanger. Analytes are likely retained on ChS-silica by hydrophilic interactions (HI) and strong cation-exchange; this was verified by investigating the effects of mobile phase composition and buffer concentration on the retention of neutral polar and basic compounds. The baseline separation of seven basic pharmaceuticals was achieved using an applied voltage of 10 kV and a mobile phase consisting of 5 mM phosphate buffer (pH 7.0) and 70% acetonitrile. A column efficiency of 153000-410000 N/m for strongly retained compounds was observed.

Keywords: Capillary electrochromatography; Chondroitin sulfate; Hydrophilic interaction; Stationary phase; Strong cation-exchange

1. Introduction
Hydrophilic interaction liquid chromatography (HILIC) is an effective separation method for polar compounds, including saccharides, amino acids, glycoproteins, nucleosides, vitamins, phenols, pesticides and toxins [1-6]. HILIC requires a hydrophilic stationary phase and an aqueous-organic solvent mobile phase with high organic solvent content, e.g., 60–95% acetonitrile (ACN). Hydrophilic interactions (HI) are based on two principles: partitioning between the bulk eluent and the water-enriched layer on the surface of the stationary phase, and the direct interactions, including hydrogen-bonding, dipole-dipole interaction and charge-dipole interaction, between the compounds and the functional groups immobilized on the stationary phase [1]. A number of silica-based stationary phases are used for HILIC, of which polysaccharides are particularly promising because their large number of hydroxyl groups enhance HI (i.e., hydrogen bonding) [7-9]. Ion exchange is another effective approach for binding charged compounds, during separation procedures, and mixed-mode HI/ ion-exchange stationary phase have been used [2,10].

Capillary electrochromatography (CEC) is a miniaturized liquid chromatographic technique that combines the high selectivity of HPLC and the high efficiency of capillary electrophoresis (CE), and it employs several packed and monolith types of stationary phases [11-14]. Recently, HI-CEC using polar stationary phases attracts an increasing attention; several columns have been developed, including silica gel, polymer bearing polar functional group and silica gel with chemically bonded polar phases [15-17]. HI-CEC column also needs the moiety which supports electroosmotic flow (EOF); therefore, a sulfonic acid group or quaternary amine group has been employed.

Chondroitin sulfate (ChS), a member of the glycosaminoglycan family is an acidic mucopolysaccharide, composed of alternating (α-1,3)-linked glucuronic acid and (α-1,4) N-acetyl galactosamine (GalNAc) unit with sulfate at either the 4- or 6- position of GalNAc [18].

ChS contains many hydroxyl groups and amide bonds

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which should promote strong HI. Furthermore, a negatively charged sulfonic acid group can support both electroosmotic flow (EOF) generation and strong cation-exchanger (SCX). Zheng et al. reported that sulfated or sulfonated cellulose derivatives immobilized on a silica stationary phase exhibited strong EOF even at low pH [19], suggesting that ChS could be an appropriate capillary electrophoresis chromatography (CEC) stationary phase for mixed-mode HI/SCX chromatography.

We here describe the preparation and characterization of a novel silica stationary phase chemically modified with ChS (ChS-silica) for HI/SCX-CEC. The ChS-silica was prepared by a simple condensation reaction between the amino group of aminopropyl silica (APS) and the carboxyl group of ChS. First, we the modification of APS with ChS was confirmed from the EOF mobilities obtained with ChS-silica at different pH values and for ChS-silica providing different elemental analyses. The mechanism of analyte separation on ChS-silica stationary phase due to HI and SCX was investigated by studying the effects of separation conditions (e.g., mobile phase composition and buffer concentration) on the retention factors ($k'$) of polar neutral and basic compounds. Finally, the efficiency of ChS-silica as a stationary phase was evaluated by chromatographing a mixture of seven basic pharmaceuticals.

2. Experimental

2.1. Chemicals

ChS C sodium salt (MW: 40000~80000, H₂O: ~10%), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinum chloride n-hydrate (DMT-MM), tetrahydrofuran (THF), formamide, pyridine, caffeine, theobromine, indapamide, trihexyphenidyl hydrochloride, hydroxyzine hydrochloride, verapamil hydrochloride, ranitidine hydrochloride and dipotassium hydrogenphosphate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Acetic anhydride, thiourea and adenine were from Kishida Chemical (Osaka, Japan). 1,7-Dimethylxanthine, cloperastine hydrochloride, alprenolol hydrochloride were from Sigma-Aldrich (St. Louis, MO, USA). 1-Methylxanthine was from Fluka (St. Gallen, Switzerland). APS (SP-120-5-APS-P) was kindly gifted from Daiso (Osaka, Japan). DIALYSIS TUBING (MW 3500) was purchased from Fisher Scientific (USA). All analytes were dissolved in water (except for indapamide dissolved in ACN) at a concentration of 1 mg/ml.

2.2. Instrumentation

All the CEC experiments were performed on a CAPI-3200 system equipped with an UV photodiode array detector (Otsuka Electronics, Osaka, Japan). Fused-silica capillaries (375 μm o.d. x 75 μm i.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). The mobile phase, prepared by mixing ACN and the phosphate buffer, was degassed thoroughly prior to use. At the beginning of each day’s works, the capillary column was conditioned with a mobile phase for 3 h and equilibrated by applying voltage 1, 3, 5 and 7 kV for 30 min each and 10 kV for 60 min. The separation voltage was set at 10 kV and the injections were made by applying a voltage of 3 kV for 3 sec. The detection wavelength was set at 200 nm, except for anthracene (EOF marker) set at 250 nm. Acetylation of ChS was confirmed by FT-IR (IR Affinity-1, Shimadzu, Kyoto, Japan) and ¹H-NMR (JIM-AL400, JEOL, Tokyo, Japan) using DMSO-d₆ (Euriso-top, France).

2.3. Stationary phase synthesis

The ChS-silica was obtained by condensation reaction of the amino group of APS and the carboxyl group of ChS, using DMT-MM which is efficient activator even in the presence of water (Fig. 1) [20]. Firstly, 0.5 g of ChS was suspended in 20 ml of THF containing 40% (v/v) water. Secondly, the carboxyl groups of ChS were activated by adding 1.0 g of DMT-MM. After 30 min, 1.0 g of APS were dispersed into the solution. The resulting mixture was stirred for 3 h at 40 °C. Finally, the reaction mixture was filtrated and intensively washed with THF/water (50%, v/v) and water. Then, the modified silica was dried under vacuum overnight.

Fig. 1. The synthesis of the ChS-silica.
2.4. Acetylated chondroitin sulfate (AC-ChS) synthesis

The AC-ChS was synthesized as previously reported [21]. Briefly, 0.5 g of dried ChS was dissolved in 5.0 ml of formamide with heating at 40 °C. To this solution, 1.6 ml of pyridine and 1.9 ml of acetic anhydride were added and the mixture was stirred for 24 h at 40 °C. A dark-brown solution was obtained and was subsequently purified by dialysis for 5 days; then, the final solution was freeze-dried. The acetylation of hydroxyl groups on ChS was confirmed by FT-IR and 1H-NMR. AC-ChS was immobilized on APS as mentioned above.

2.5. Column preparation

CEC column packed with the ChS-silica were prepared by the same slurry packing method as our previous reports [11]. After packing of the ChS-silica by an HPLC pump, the slurry solvent in the capillary column was replaced with water and outlet frit was made at 250 mm from inlet frit by sintering with a hot resistance wire. The polystyrene coating was then burned away to make a detection window at 5 mm from outlet frit. All columns were 360 mm long with a packed length of 250 mm. The prepared capillary column was initially flushed with mobile phase at 30 bar with an HPLC pump for 2 h. Then, elevated voltage from 1 to 10 kV was applied to the capillary column for equilibration.

3. Results and discussion

3.1. Characterization of the ChS-silica column

The modification of APS by ChS was characterized from the EOF mobilities obtained with ChS-silica at different pH values and for ChS-silica yielding different elemental analyses. APS produces an anodic EOF due to its positive amino groups, so modification of APS with ChS should result in a cathodic EOF over a wide range of pH value due to the presence of sulfonic acid groups (pKa = 0.699 [22]).

The EOF mobility (2.1x10^8 mm/Vs) of the ChS-silica column remained constant at the three experimental pH values tested (3.0, 5.0 and 7.0) and comparable to that of other silica materials bearing sulfonic acid groups [22-25]. The results of elemental analysis following modification (C: 8.83%, N: 1.58%, H: 1.22%) compared to before modification (C: 3.90%, N: 1.10%, H: 0.27%) allowed the modification ratio of ChS onto APS, (determined to be 135 mg/g) to be calculated from N.

ACN concentration of 60-95% are typically used for HILIC but this high content of organic solvent induces bubble formation in the CEC column due to Joule heating [13,14]. Therefore, the effect of 50%, 60% and 70% ACN on the retention of polar neutral compounds (caffeine, 1-methylxanthine, 1,7-dimethylxanthine and theobromine) was investigated by keeping the phosphate buffer (pH 7.0) concentration at 5 mM. As predicted from the mechanism underlying HI, the retention factors increased with an increase in ACN concentration. Xanthines remain neutral under the experimental pH value (pH 7.0) [10,26], and thus their retention likely does not involve electrostatic or charge-dipole interactions. The xanthines were separated on a ChS-silica column with 91000 to 115000 N/m theoretical plates (Fig. 2).

The chromatographic retention of charged compounds on ChS-silica is expected to also involve SCX, which can be modulated by the buffer concentration. The effect of buffer concentration in the mobile phase on the retention of three basic compounds (adenine, adenosine, and N,N-diethylaniline) was studied by changing phosphate buffer (pH 7.0) concentration (Fig. 3). All the retention factors decreased with increasing buffer concentration, consistent with the principles underlying SCX. In contrast, HI increases as the buffer concentration of the mobile phase
increases because high salt concentration drives salt ions into the water-rich liquid layer, increasing the volume and hydrophilicity of the liquid layer [8]. The result obtained using the ChS-silica column indicate that the retention and separation of basic compounds is mainly governed by SCX, rather than HI; nonetheless, even the weak dual nature of the column resulted in basic compounds being retained more strongly than polar neutral compounds (xanthines and thiourea) under the same mobile phase condition.

The role of hydroxyl groups in the HI-CEC mode retention by using acetylated ChS-silica (AC-ChS-silica) column. The modification ratio of AC-ChS-silica (132 mg/g) was calculated by the elemental analysis (C: 9.18%, N: 1.42%, H: 0.76%). The retention factors of xanthines on the AC-ChS-silica column decreased by 30-60% compared with the ChS-silica column, suggesting that the hydroxyl groups of ChS were the primary contributors to HI.

3.2. Separation of basic pharmaceuticals

The separation of basic compounds by CEC can be challenging due to their electrostatic adsorption resulting in incomplete elution and/or peak tailing [23,27]. Baseline separation of seven basic pharmaceuticals was obtained on the ChS-silica column without peak tailing and with extremely high column efficiencies between 83000 to 408000 N/m (Fig. 4 and Table 1). A capillary zone electrophoresis experiment showed that all analytes except indapamide were positively charged under the mobile phase condition and showed column efficiencies higher than 100000 N/m. Several researchers have reported extremely high efficiencies of positively charged compounds on CEC columns packed with SCX particles [25,28,29] and Smith et al. suggested that some form of sample stacking mechanism may underlie this peak sharpening effect [28]. Additionally, the residual amino groups on ChS-silica may suppress peak tailing [27].

![Fig. 4. Separation of basic pharmaceuticals on the ChS-silica column. Conditions as in Fig. 2. Peaks: 1, indapamide; 2, cloperastine; 3, trihexyphenidyl; 4, hydroxyzine; 5, alprenolol; 6, verapamil; 7, ranitidine.](image)

Table 1. Retention factor, pKa and plate number of basic pharmaceuticals.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>k'</th>
<th>pK a</th>
<th>N/m</th>
</tr>
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<tbody>
<tr>
<td>indapamide</td>
<td>0.04</td>
<td>9.35</td>
<td>83000</td>
</tr>
<tr>
<td>cloperastine</td>
<td>3.09</td>
<td>8.69</td>
<td>410000</td>
</tr>
<tr>
<td>trihexyphenidyl</td>
<td>3.42</td>
<td>9.31</td>
<td>153000</td>
</tr>
<tr>
<td>hydroxyzine</td>
<td>3.46</td>
<td>6.62</td>
<td>361000</td>
</tr>
<tr>
<td>alprenolol</td>
<td>3.97</td>
<td>9.43</td>
<td>326000</td>
</tr>
<tr>
<td>verapamil</td>
<td>4.07</td>
<td>8.97</td>
<td>284000</td>
</tr>
<tr>
<td>ranitidine</td>
<td>6.31</td>
<td>8.35</td>
<td>226000</td>
</tr>
</tbody>
</table>

Table 2. Repeatability and reproducibility of separation parameters of the ChS-silica columns expressed as RSD % of EOF mobility, retention factor, and plate number.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Repeatability and reproducibility (RSD %)</th>
<th>Run-to-run (n=3)</th>
<th>Column-to-column (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOF mobility</td>
<td>2.9</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>k'</td>
<td>≤2.3</td>
<td>≤5.2</td>
<td></td>
</tr>
<tr>
<td>N/m</td>
<td>≤14.0</td>
<td>≤28.6</td>
<td></td>
</tr>
</tbody>
</table>

CEC conditions: mobilephase, 5 mM phosphate buffer (pH 7.0)/ACN = 30/70 (v/v, %); voltage, 10 kV; injection, 3 kV for 3 s; Test compounds: same as Table 1.

In accordance with previous work [30], the repeatability of the analysis and the reproducibility of the preparation of capillary columns packed with ChS-silica were studied using three columns. The repeatability and reproducibility, expressed as relative standard deviations (RSDs) of the chromatographic parameters (i.e., EOF mobility, retention factors (k') and plate number (N/m)), were measured using basic compounds (Table 2) and demonstrate a practical application of ChS-silica for HI-CEC.

4. Conclusions

ChS-silica was synthesized and its utility for CEC separations was evaluated. The sulfonic acid groups on ChS-silica support substantial and stable EOF over a wide range of buffer pH values for CEC. Polar and basic compounds were used to evaluate the chromatographic properties of the ChS-silica column and its HI/SCX mixed mode retention mechanism was demonstrated. Furthermore, evaluation of the AC-ChS column confirmed that ChS hydroxyl groups play an important role in HI retention. Strong basic pharmaceuticals were separated with extremely high efficiency on the ChS-silica column. CEC with ChS-silica will be useful for the separation of a variety of basic compounds such as alkaloids and peptides.
References


