Silica based click amino stationary phase for ion chromatography and hydrophilic interaction liquid chromatography†

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A silica based amino stationary phase was prepared by immobilization of propargylamine on azide-silica via click chemistry. This readily prepared click amino stationary phase demonstrated good selectivity in separation of common inorganic anions under ion chromatography (IC) mode, and the triazole ring in combination with free amino group was observed to play a major role for separation of the anions examined. On the other hand, the stationary phase also showed good hydrophilic interaction liquid chromatography (HILIC) properties in the separation of polar compounds including nucleosides, organic acids and bases. The retention mechanism was found to match well the typical HILIC retention.

Introduction

Since ion chromatography (IC) was described by Small et al. in 1975, it has been a well-established technique for the analysis of ionic samples especially to inorganic anions. As a key component, stationary phases have always been paid much attention. Among them, polymer-based ones are widely used in present IC system due to their wide pH tolerance. Although the silica-based ones have limited pH tolerance, they have some desirable features such as high efficiency and good mechanical stability. In addition, the rich silanol groups onto the surface of silica gel allow easy introduction of many functional groups onto the gel. On the other hand, hydrophilic interaction liquid chromatography (HILIC) has been a powerful separation technique suitable for polar and hydrophilic compounds under HILIC or mixed mode.3–5 The polar functional groups existing in the stationary phase for HILIC often find new applications in IC.6–9 A poly(ethylene oxide)-bonded stationary phase, commercially available for HILIC, demonstrated good separation of inorganic anions.6 Similarly, a pyridine stationary phase for HILIC was observed to be efficient for separation of inorganic anions and ion exchange dominated the separation process.7 In addition, quaternary ammonium, sulfonyl and zwitterionic group-functionalized polymers or silica beads were also used for analysis of ionic compounds.8–12 Recently a variety of HILIC stationary phases have been developed for different purposes, such as separation of polar compounds and peptides by multi-dimensional separation techniques,13–20 enrichment of lower abundance biological compounds by HILIC solid-phase extraction (SPE), including glycopeptides, proteins and glycoproteins,21–23 as well as analysis of complicated polar samples by using HILIC-MS hyphenated technique, due to good compatibility between organic mobile phase used in HILIC with MS.24 A common characteristic between IC and HILIC stationary phases is that both contain polar or hydrophilic groups. In IC, ion exchange occurs between charged or chargeable moieties of the stationary phase and the ionic analytes, while HILIC displayed both electrostatic interaction and partition interaction between stationary phase and analytes. Based on these considerations, we are trying to develop a new stationary phase which could be used for IC and HILIC.

Cu(i) catalyzed alkynyl-azide 1,3-dipolar cycloaddition (CuAAC) has been proven to be a powerful strategy for immobilized functional groups on different support materials, and a series of silica based stationary phases have been prepared via this method.17,18,25–28 It is noticeable that a regioselective 1,2,3-triazole ring is formed in this reaction. In previous work, we have described a simple hydrophilic stationary phase bearing a 1,2,3-triazole ring and a hydroxymethyl group in 4-position of this ring, which could be used for separating polar compounds under HILIC mode as well as separating anions under IC mode.18,29 The existence of 1,2,3-triazole ring plays a major role in the separation of inorganic anions.29 A drawback of this stationary phase is the limited exchange capacity. To address this, herein, a novel click triazole-amino stationary phase (CTASP) was designed and readily prepared via CuAAC reaction between the azide silica and 3-aminopropyne. The application of this new stationary phase for IC and HILIC will be explored.

Experimental

Apparatus and reagents

Chromatographic grade acetonitrile, formic acid and ammonium formate were purchased from Tedla (USA). Ammonium formate...
and copper iodide were from Acros (USA). Other reagents were domestic and used without further purification. The chromatographic experiments were performed on Agilent 1200 series and a Waters HPLC system consisting of a 515 pump, a 5 μL Rheodyne injector and a 2487-UV absorbance detector. Milli-Q ultrapure water was used throughout unless stated.

Preparation of the CTASP and amino column

Spherical silica was from Fuji (5 μm particle size; 10 nm pore size; 300 m² g⁻¹; surface area, Fuji Silysia Chemical Ltd., Japan). The preparation of azide 3-azide-propyl triethoxysilane silylation reagent was described previously. 2-Propynylamine was subsequently bonded to silica beads via click chemistry. A suspension of 3-azidepropyl triethoxysilane (7.50 g) and silica beads (5.00 g) in toluene was refluxed for 36 h, then the silica beads were collected by filtration to yield azide silica bead (5.6 g). The 2-propynylamine was covalently bonded to silica beads as follows: the azide silica beads (3.00 g), 2-propynylamine (1.65 g) and CuI (0.57 g) was added in 100 mL toluene, then the suspension was slowly stirred at 45 °C for 24 h. After reaction, all solid materials were collected by filtration and washed well with toluene (200 mL), acetone (300 mL), methanol (100 mL) and THF (500 mL), respectively, and then got the CTASP. See synthesis diagram of CTASP was provided in Fig. 1.

For comparison, the common amino stationary phase (ASP) was also prepared. Briefly, a suspension of activated silica beads (4 g) and 3-aminopropyl triethoxysilane (1.6 mL) in toluene (80 mL) was refluxed for 24 h, all the solid materials were collected by filtration, and washed well with toluene (200 mL), THF (200 mL), methanol (200 mL), water (300 mL) and methanol (200 mL) in turn.

After the CTASP and ASP were dried under vacuum, the stationary phase was slurry-packed into stainless-steel column (100 mm long × 4.6 mm i.d.) with ethanol as slurry solvent and propulsion solvent.

Measurement of effective ion exchange capacity of CTASP

The effective ion exchange capacity of CTASP was estimated through the breakthrough method. Briefly, the column was flushed with 50 mM NaCl eluent for 2 h at 1 mL min⁻¹, pure water for 1 h at 1 mL min⁻¹. Finally the absorbed Cl⁻ was released by using 5 mM NaNO₃ solution at 0.4 mL min⁻¹, at the same time the effluent was monitored by UV absorbance detector operated at 210 nm until the breakthrough curve was achieved. The column capacity (Q) could be calculated by the equation $Q = CF(t_b - t_o)$. In the equation, C is the concentration of NaNO₃ eluent (mol L⁻¹), F is the flow rate of NaNO₃ eluent (mL min⁻¹), $t_b$ is the time at 100% breakthrough of NaNO₃ (min), and $t_o$ is the void time of the column (min).

Results and discussion

Characterization of CTASP

The azide-silica and CTASP was characterized by FT-IR and elemental analysis, as shown in support information SI-Fig.1 and SI-Table.† A peak for the azide stretch at 2112 cm⁻¹ disappeared after the “click” step, indicating that the azide had reacted efficiently with alkynyl via click chemistry. The increase of carbon and nitrogen content demonstrated that propargylamine was bonded to azide-silica successfully. The apparent anion exchange capacity determined from the average nitrogen percentage obtained by elemental analysis was ~1.44 mmol g⁻¹. While for the tested column, its effective capacity was measured to be 92 µmol column⁻¹ through the breakthrough method mentioned above. In comparison, the apparent exchange capacity for ASP was determined to be ~0.74 mmol g⁻¹ from the average nitrogen percentage (provided in SI-Table†).

Chromatographic evaluation of CTASP under IC mode

It has been proven that 1,2,3-triazole with an adjacent hydroxyl group has good separation of inorganic anions. While its drawback was limited effective anion capacity (column, 4.6 mm i.d. × 150 mm long, 2.67 μmol column⁻¹). The CTASP, 1,2,3-triazole with an adjacent amino group, demonstrated much higher capacity (column, 4.6 mm i.d. × 100 mm long, 92 μmol column⁻¹). To evaluate the performance of CTASP for IC, several inorganic anions with UV absorption were chosen to be the model sample and 5 mM Na₂SO₄ + 0.5 mM H₂SO₄ was used to be the eluent. A typical chromatogram is provided in Fig. 2. Five inorganic anions including IO₃⁻, BrO₃⁻, Br⁻, NO₃⁻ and I⁻ were well separated in a short time (<9 min) with good peak shape and high efficiency, e.g., the plate numbers of Br⁻ and NO₃⁻ were calculated to be ~71 000 and 74 000 plates m⁻¹, respectively. The plot between the logarithm of the retention factor (log k) of the analytes versus the logarithm of the Na₂SO₄ concentration was highly linear. The slopes were ~0.487, ~0.477, ~0.462, ~0.435 and ~0.417 for IO₃⁻, BrO₃⁻, Br⁻, NO₃⁻ and I⁻, respectively, which were in agreement with the theoretical slope of ~0.5 for anion exchange elution of a singly charged anion (NO₃⁻) with a double-charged eluent anion (SO₄²⁻). This indicates that the ion exchange mechanism dominate the separation process. In addition, run-to-run reproducibility of CTASP was measured by consecutive injections (see support information SI-Fig. 2†). Their relative standard deviation (RSD) of retention times (and peak area) over 12 consecutive runs were 0.12% (and 5.34%), 0.12% (and 4.1%), 0.11% (and 3.87%), 0.15% (and 3.9%), 0.29% (and 5.12%) for IO₃⁻, BrO₃⁻, Br⁻, NO₃⁻ and I⁻ respectively. In addition, the column-to-column reproducibility was measured to be ~5.8% (n = 3) in terms of retention time of NO₃⁻, indicating good reproducibility of CTASP. This mainly results from its simple chemical structure and ease of preparation procedure.

In order to highlight the role of 1,2,3-triazole for the separation of anions, CTASP and ASP were compared under the same conditions. The results show that five inorganic anions were well...
separated on CTASP, while ASP demonstrated poor separation and only three peaks were observed for five anions due to much less retention (shown in Fig. 2). Obviously, 1,2,3-triazole rings play an important role in the separation of anions. As indicated previously, the protonated triazole ring or trapped eluent cation served to be ion exchange sites. In the eluent of 5 mM Na$_2$SO$_4$ + 0.5 mM H$_2$SO$_4$ and 5 mM Na$_2$SO$_4$ were used to evaluate the retention of the analytes, respectively, as provided in support information SI-Fig.3† The results show that the retention of five model anions slightly increased with the decrease of the eluent pH value, indicating that the pH value of the eluent has less of an effect on the retention of the analytes. This is consistent with the previous reports in which the pH value of the eluent had less of an effect on the retention of anions in HILIC with an adjacent hydroxyl group-based stationary phase. One interesting thing should be noted, the retention order of anions on ASP is much different with CTASP. More specifically, IO$_3^-$, which is always much less retained on common anion exchanger including CTASP, was observed to be strongly retained on ASP column, it eluted after NO$_3^-$ and even I$^-$ (normally strongly retained on common anion exchangers). Presently we do not know the exact reason for this unusual behavior and further study is under way.

The utility of the CTASP was demonstrated to determine the inorganic anion in the tap water. The concentrations of nitrate in the tap water were computed to 0.211 mM according to the calibration equation achieved in the concentration ranging from 0.01–0.2 mM. (Note: the real sample was diluted to 5-fold with pure water for injection. Since UV absorption detector was employed in this study, thus only those anions with enough UV absorption could be detected. In addition, here we just want to demonstrate the utility of the proposed column, no optimization of the experimental conditions has been made to improve the detection sensitivity.) The repeatability of the peak height for nitrate was found to be 1.84% for three consecutive runs and the spiked recovery of nitrate was ∼95%.

**Chromatographic evaluation of CTASP under HILIC mode**

HILIC has been a good separation technique which is well suited to the separation of polar or hydrophilic compounds. Limited kinds of commercially available stationary phases have restricted its wide applications to some extent. Commercial amino stationary phase has been reported to perform well under HILIC mode. Herein the performance of CTASP under HILIC mode was explored to separate nucleosides, small molecule organic acids and organic bases. The data are provided in Fig. 3. From Fig. 3A, 8 nucleosides could be well separated with good peak shape. Baseline separation of 5 organic acids and 4 organic bases could also be achieved (as shown in Fig. 3B). Note: to facilitate the comparison, the signal polarity of organic bases was deliberately inverted to be negative.

For further understanding the chromatographic retention characteristics of CTASP the effect of water content in mobile phase on retention behaviors of nucleosides was investigated by varying the water content in the mobile phase (ACN/H$_2$O). The capacity factors of model compounds were plotted against the water content in the mobile phase, as shown in Fig. 4. It can be seen that typical HILIC behaviors of this CTASP were displayed, all retention of analytes decreased with the increase of water content in the mobile phase. In addition, similar behavior for small molecule acids was also observed (data not shown).

The addition of electrolyte to the mobile phase in HILIC mode is always used to improve the selectivity and column efficiency. Due to high content of organic solvent in the mobile phase (commonly >60%), good compatibility of the solubility between the added electrolyte and the organic solvent is required; ammonium formate or ammonium acetate is commonly used as the added electrolyte in HILIC. Here the effect of salt concentration of mobile phase on the retention was also explored by varying ammonium formate concentration from 5 to 20 mM in the mobile phase (provided in support information SI- Fig. 4†). In the case of nucleosides, retention of all analytes increased with the increase of salt concentration due to the enhancement of the polarity of the rich water layer resulting from high salt concentration. However, for small molecular organic acids, the effect of salt concentration appears to be complex. At low concentration section, the retention decreased with the increase of salt concentration, which could be probably explained by ion exchange mechanism dominating the separation process. While at high concentration section, the retention slightly increased with the increase of salt concentration, indicating that HILIC mechanism dominates the separation process due to the enhancement of the polarity of the rich water layer. In addition, the effect of pH value of the mobile phase was also explored. In the tested pH range of 3.2–5.2, the data achieved indicated that the slight increase of retention factor was observed, except cytidine ($pK_a = 4.6$). Similarly, there was less dependent of the retention of organic bases on the pH value of the mobile phase. In comparison, there was significant increase of retention factor for tested small molecule acids, as shown in support information.
SI-Fig. 5† It can be explained that the dissociation of small molecule acids was enhanced with the increase of pH value of the mobile phase, then leading to strong interaction with the stationary phase due to strong ion exchange interaction.

Conclusion
A novel CTASP was prepared via click chemistry and its chromatographic behaviors were investigated under both IC and HILIC mode. The stationary phase has a very simple structure and is easy to prepare. Inorganic anions could be well separated under IC mode, and the polar compounds including nucleosides, organic acids and bases could also be well separated under HILIC mode. Further understanding its chromatographic retention mechanisms and broadening application are still under way.

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Fig. 3 Chromatograms of nucleosides, organic bases and acids on CTASP-based column. Conditions: eluent, 90% ACN/10% H2O (100 mM HCOONH4, pH 3.15); UV, 254 nm; other conditions same as in Fig. 2; Peak identification: A, *Unknown peak; (1) sulfadiazine; (2) uracil; (3) 5-methyl uracil; (4) uridine; (5) adenosine; (6) adenine; (7) cytosine; (8) cytidine; B, organic bases separation, b-1, diphenylamine; b-2, caffeine; b-3, theophylline; b-4, diprophyllline; organic acids separation: a-1, salicylamide; a-2, benzoic acid; a-3, acetyl salicylic acid; a-4, salicylic acid; a-5, 4-nitro salicylic acid.

Fig. 4 Effect of water content in the mobile phase on the retention of nucleosides. Conditions: sample, nucleosides; other conditions the same as in Fig. 3.

References