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Glutathione-based zwitterionic stationary phase for hydrophilic interaction/cation-exchange mixed-mode chromatography

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ABSTRACT

As a naturally hydrophilic peptide, glutathione was facilely immobilized onto silica surface to obtain a novel hydrophilic interaction/cation-exchange mixed-mode chromatographic stationary phase (Click TE-GSH) via copper-free "thiol-ene" click chemistry. The resulting material was characterized by solid state ^{13}C/CP MAS NMR and elemental analysis. The measurement of ζ-potential indicated the cation-exchange characteristics and adjustable surface charge density of Click TE-GSH material. The influence of acetonitrile content and pH value on the retention of ionic compounds was investigated for understanding the chromatographic behaviors. The results demonstrated that Click TE-GSH column could provide both hydrophilic and cation-exchange interaction. Taking advantage of the good hydrophilicity and inherent cation-exchange characteristics of Click TE-GSH material, the resolution of neutral fructosan with high degree of polymerization (DP), basic chitooligosaccharides and strongly acidic carrageenan oligosaccharides was successfully realized in hydrophilic interaction chromatography (HILIC), hydrophilic interaction/cation-exchange mixed-mode chromatography (HILIC/CEX), cation-exchange chromatography (CEX) and electrostatic repulsion/hydrophilic interaction chromatography (ERLIC). On the other hand, the separation of standard peptides varying in hydrophobicity/hydrophilicity and charge was achieved in both CEX and HILIC/CEX mode with high efficiency and distinct selectivity. To further demonstrate the versatility and applicability of Click TE-GSH stationary phase, the separation of a human serum albumin (HSA) tryptic digest was performed in HILIC/CEX mode. Peptides were adequately resolved and up to 86 HSA peptides were identified with sequence coverage of 85%. The results indicated the good potential of Click TE-GSH material in glycomics and proteomics.

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1. Introduction

The investigation of hydrophilic interaction chromatography [1] (HILIC) has attracted more and more attention owing to its special capability and versatility in the separation of hydrophilic and ionic compounds [2–11], such as polar drugs, metabolites, amino acids, peptides, carbohydrates and so on. Nonetheless, the development of novel HILIC stationary phase with higher hydrophilicity and better selectivity is still of great importance for its applications, especially in the resolution of oligosaccharides with high degree of polymerizations (DPs) and complex peptide mixtures in proteomics. According to previous reports, ionic interaction superimposed on hydrophilic interaction can effectively improve the separation selectivity [12–14]. The combination of hydrophilic and anion-cation exchange interaction, i.e., mixed-mode anion–cation exchange/hydrophilic interaction liquid chromatography (ACE/HILIC) proved to be an efficient technology for the analysis of small molecule drug from both biological and synthetic sources of molecular diversity [14]. Hodges et al. have demonstrated that the combination of hydrophilic interaction and cation-exchange interaction, i.e., hydrophilic interaction/cation-exchange mixed-mode chromatography (HILIC/CEX) is well complementary to RPLC for the separation of peptides, including linear peptides, synthetic amphipathic α-helical peptides, etc. [12,15–17]. Lindner et al. also highlighted the potential of mixed-mode HILIC/CEX for the resolution of proteins [18,19].

Zwitterionic stationary phases [20,21], which contain both positive and negative charges on the surface, have been widely used in HILIC [10,22–24]. In the early reports [25,26], we have designed and synthesized cysteine-based zwitterionic HILIC stationary phase (Click TE-Cys) based on copper-free "thiol-ene" click chemistry [27–29] between vinyl silica and thiol group on cysteine. The introduction of electrostatic interaction and multipoint intermolecular

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interaction is considered to be advantageous for improving separation selectivity. However, zwitterionic materials developed for HILIC, including the typical sulfobetaine/phosphorylcholine-based materials and Click TE-Cys material, exhibit slight surface charge and limited structures for multipoint interaction.

As naturally zwitterionic compounds, peptides can display good hydrophilicity, inherent ionic-exchange characteristics and various spatial structures, which represent a good choice as bonded components. Among the natural peptides, glutathione (GSH) which has multiple biological functions [30,31] is a highly hydrophilic tripeptide composed of glutamic acid, cysteine and glycine. It possesses two free carboxylic acid groups and one amino group, and should exhibit cation-exchange characteristics if immobilized. Meanwhile, GSH possesses abundantly polar groups which might provide stronger intermolecular interaction, such as hydrogen bonding interaction. Accordingly, GSH, which contains a thiol group, was immobilized onto vinyl silica via "thiol-ene" click chemistry to obtain a HILIC/CEX mixed-mode chromatographic stationary phase (designated as Click TE-GSH). The resulting stationary phase can be successfully applied in HILIC, HILIC/CEX and cation-exchange chromatography (CEX). The ζ-potential on Click TE-GSH material was characterized to understand the surface charge properties. The influence of acetonitrile content and pH value in the mobile phase on the retention of small polar compounds was carried out to investigate the retention behaviors of Click TE-GSH stationary phase. In light of the good hydrophilicity and inherent cation-exchange characteristics of Click TE-GSH stationary phase, the application in the separation of neutral fructosan, basic chitooligosaccharides and strongly acidic carrageenan oligosaccharides was performed in HILIC, CEX and mixed-mode hydrophilic interaction/ion-exchange chromatography (HILIC/IEX). Additionally, the resolution of model peptides and a complex human serum albumin (HSA) tryptic digest was successfully realized on the Click TE-GSH mixed-mode stationary phase.

2. Experimental

2.1. Materials

Spherical silica (5 μm particle size, 100 Å pore size, 300 $m^2 g^{-1}$ surface area) was purchased from Fuji Silysia Chemical (Kasugai, Japan). Trichlorovinylsilane was obtained from ABCR (Karlsruhe, Germany), α,α′-azodiisobutyronitrile (AIBN) was purchased from Shanghai Chemical Reagents (Shanghai, China) and glutathione (GSH) was from Acros (Fair Lawn, NJ, USA). C18 material for desalting was from Acchrom Co., Ltd. (Beijing, China). GELoader tips were obtained from Eppendorf (Hamburg, Germany). The standard peptides KRQYKSILQEENRR, NRAESFRQLWDGARK, GFGRYRRHGSPW, DAEFRHDSGYE, FAGLRQAVTQGFPTEL and EGVNDNEEGFFSAR were obtained from ChinaPeptides Company (Shanghai, China). Fructosan, chitooligosaccharides and carrageenan oligosaccharides were all kindly donated by the Natural Products and Glycobiotechnology group (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, PR China). Polar solutes uridine, cytosine, cytidine, adenine, adenosine, inosine, salicylic acid, orotic acid and propranolol were from Acros. Melamine was from Tianjin Chemical Reagents (Tianjin, China). Clenbuterol, ractopamine and salbutamol were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile (CH_3CN) and methanol (MeOH) of HPLC grade was from Merck (Darmstadt, Germany). Water (H_2O) was purified by a Milli-Q water purification system (Billerica, MA, USA). All other reagents were analytical grade reagents and used without purification.

Silica column (4.6 mm × 150 mm, 5 μm, 100 Å) was from Acchrom (Beijing, China), ZIC®-HILIC (2.1 mm × 150 mm, 3.5 μm, 100 Å) and ZIC®-cHILIC (4.6 mm × 150 mm, 3 μm, 100 Å) were purchased from Merck (Darmstadt, Germany).

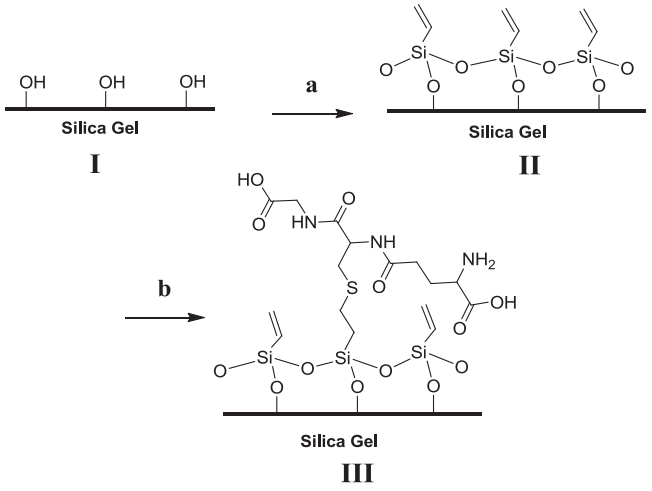

Fig. 1. The preparation of zwitterionic stationary phase (Click TE-GSH) based on thiol-ene click chemistry. (a) Toluene, trichlorovinylsilane, room temperature; (b) MeOH/H_2O (5:7, v/v), glutathione, AIBN, 65 °C.

2.2. Apparatus

All the chromatographic evaluation was performed on an Alliance HPLC system equipped with a Waters 2695 HPLC pump, a Waters 2996 diode array detector (DAD) and a Waters 2424 evaporative light scattering detection (ELSD) system (Waters, Milford, MA, USA). Chromatograms were recorded using Empower workstation software. Elemental analysis was performed on a Vario EL III elemental analysis system (Elementar, Hanau, Germany). Solid state ^{13}C cross polarization/magic-angle spinning (CP/MAS) NMR characterization was performed on a Varian InfinityPlus 400 NMR Spectrometer (400 MHz, 9.39 T) (Palo Alto, CA) and the chemical shifts of ^{13}C were referenced to tetramethylsilane (TMS). The ζ-potential was measured on a Malvern Zetasizer Nano-ZS90 instrument (Malvern, UK) according to the literature [22]. Stock sample solution was prepared by suspending 50 mg of material in 30 mL of water. And the final samples were prepared by mixing 1 mL of stock sample with 2 mL of ammonium formate buffer (100 mM), and then diluted with water to 10 mL. After a solution was made, it was thoroughly mixed and immediately transferred to the measurement cell. All the separation experiments were repeated at least once except for the separation of the HSA tryptic digest. The time required for column equilibration was 20 min (or 15 min) for mobile phase with (or without) buffer solution.

2.3. Preparation of HILIC/CEX mixed-mode stationary phase based on GSH

The synthesis of GSH-bonded stationary phase was shown in Fig. 1. Vinyl silica **II** was prepared according to the procedure as described in our previous paper [25]. After that, 10 g of the vinyl silica **II** was added into the solution of GSH (3 g) in 120 mL of water–methanol (7:5, v/v) under nitrogen atmosphere. 160 mg of AIBN was then added into the stirred solution as catalyst and the reaction was continued for 48 h at 65 °C. The resulting material was filtered, washed successively with water and methanol and then dried at 80 °C overnight. The obtained stationary phase **III** was designated as Click TE-GSH ("Click TE" represents "thiol-ene" click chemistry).

With 40 mL of methanol as slurry solvent and 80 mL of methanol as propulsion solvent under a pressure of 60 MPa, 2.0 g of the

resulting Click TE-GSH material was slurry-packed into stainless steel column (150 mm × 4.6 mm I.D.). The void time of Click TE-GSH column was measured using toluene as t_0 maker and the resulting t_0 was 1.75 min. The retention of cytosine and cytidine remained almost unchanged on Click TE-GSH column after 6 months of utilization.

2.4. MS identification of peptides

2.4.1. Desalting

Each peptide fraction eluted from Click TE-GSH column was dried by lyophilization and redissolved in H_2O/FA (100:0.1, v/v, 200 μL). C18 material (about 1 mg) was packed into the GELoader tip with CH_3CN as slurry solvent. The microcolumn was activated with CH_3CN (20 μL) and equilibrated with H_2O/FA (100:0.1, v/v, 20 μL). Then each fraction (20 μL) was loaded onto the column. After rinsing with H_2O/FA (100:0.1, v/v), CH_3CN/H_2O/FA (50:50:0.1, v/v/v, 10 μL) was utilized for elution and the peptide fraction was collected.

2.4.2. Nano-electrospray ionization-MS analysis

Peptides were analyzed on a Waters Acquity nano-LC system (Milford, MA, USA) coupled to nano-electrospray ionization-quadrupole time-of-flight (ESI Q-TOF) mass spectrometer (Waters, Manchester, UK). The desalted peptide fraction was directly infused into the nano-ESI source operating under positive ion mode with nanospray voltage at 2.3 kV. MS data was acquired at m/z 500–2000. Peptides were identified manually by comparing the theoretical mass and charge of tryptic human serum albumin digests with the observed m/z.

3. Results and discussion

3.1. Synthesis and characterization of the HILIC/CEX mixed-mode material

As shown in Fig. 1, the preparation of Click TE-GSH stationary phase was based on the copper-free "thiol-ene" click chemistry. Compared with the typical Cu(I)-catalyzed azide-yne Huisgen reaction [27,32], the utilization of thiol-ene click reaction can effectively avoid residual metal ions in the chromatographic packings. Click TE-GSH material was characterized by solid state ^{13}C/CP MAS NMR (Fig. 2a). The signal at 173.4 ppm was assigned to the carbon atoms of four carbonyl groups. The peaks marked with asterisks (233.4 ppm and 113.6 ppm) belonged to the sidebands of the peak at 173.4 ppm. The resonances at 137.9 ppm and 129.8 ppm were assigned to the carbon atoms of unreacted vinyl groups on the stationary phase. The signals at 12.1 ppm and 18.8 ppm belonged to the carbon atoms of reacted vinyl groups. The resonances between 16.8 ppm and 54.2 ppm belonged to the carbon atoms of GSH (Fig. 2b). The result indicated that GSH has been successfully bonded onto silica surface. Meanwhile, elemental analysis was carried out for characterization of vinyl silica **II** and Click TE-GSH material **III**. The carbon content and nitrogen content were 4.45% and 0.00% for vinyl silica **II**, while for Click TE-GSH material **III** were 9.43% and 1.93%, respectively. The surface bonding density calculated according to the increase of carbon content [33] was 1.58 μmol/m^2. The ζ-potential of Click TE-GSH material was measured at different pH values (pH 2.5–6.5) according to the literature [22] in order to understand the surface charge properties. Though Click TE-GSH has a zwitterionic pair, it also has a free carboxylic group at the C-terminus of the glycyl-residue of GSH. Consequently, it showed negative surface charge within the pH range of 3.0–6.5 (Fig. 3), demonstrating its cation-exchange characteristics.

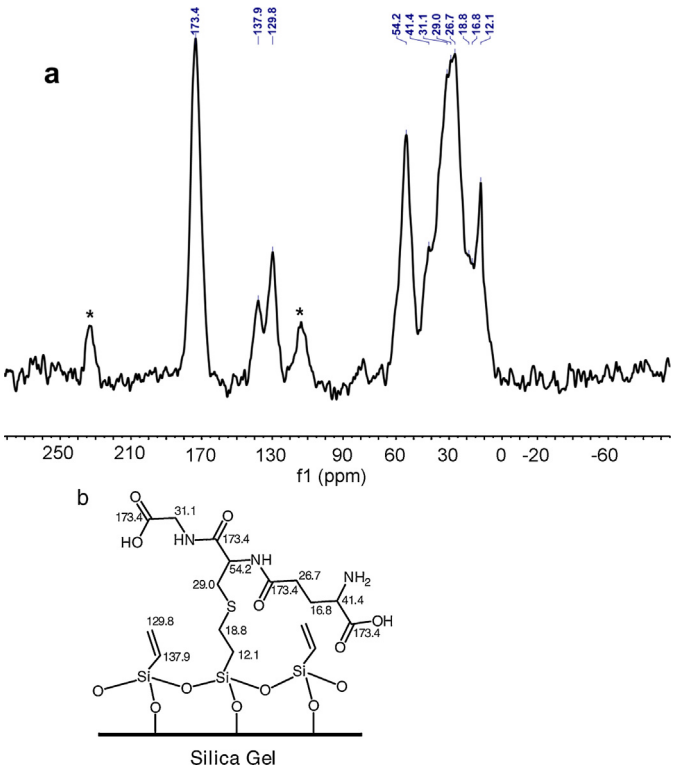

Fig. 2. Solid state ^{13}C CP/MAS NMR spectra of Click TE-GSH material.

The surface charge density on Click TE-GSH material could be easily regulated by changing pH value in the mobile phase.

3.2. Chromatographic characteristics of Click TE-GSH stationary phase

To understand the retention behaviors of Click TE-GSH material, the influence of CH_3CN content on the retention of four small polar compounds (structures shown in Fig. S1, SI) was investigated. As shown in Fig. 4, the retention of adenine (pK_a 4.2, 9.8) decreased with the decrease of CH_3CN content from 90% to 50%, which presumably was influenced by hydrophilic partitioning mechanism. The retention increased with the decrease of CH_3CN content from 50% to 10%, which was caused by the increasing electrostatic attraction between the basic compound and negatively charged

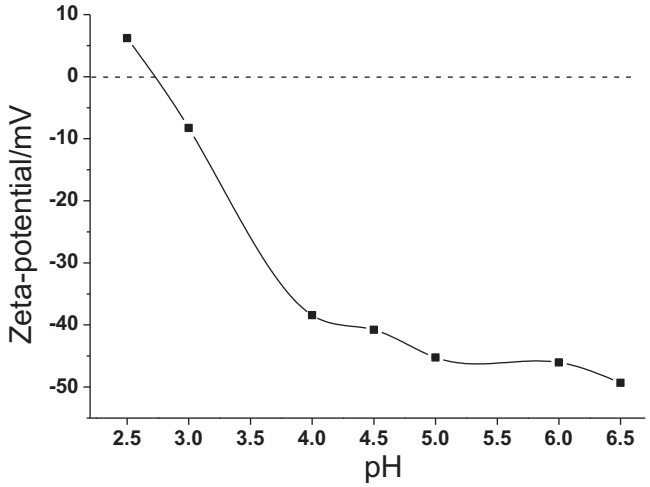

Fig. 3. The ζ-potential measurements on the zwitterionic Click TE-GSH material.

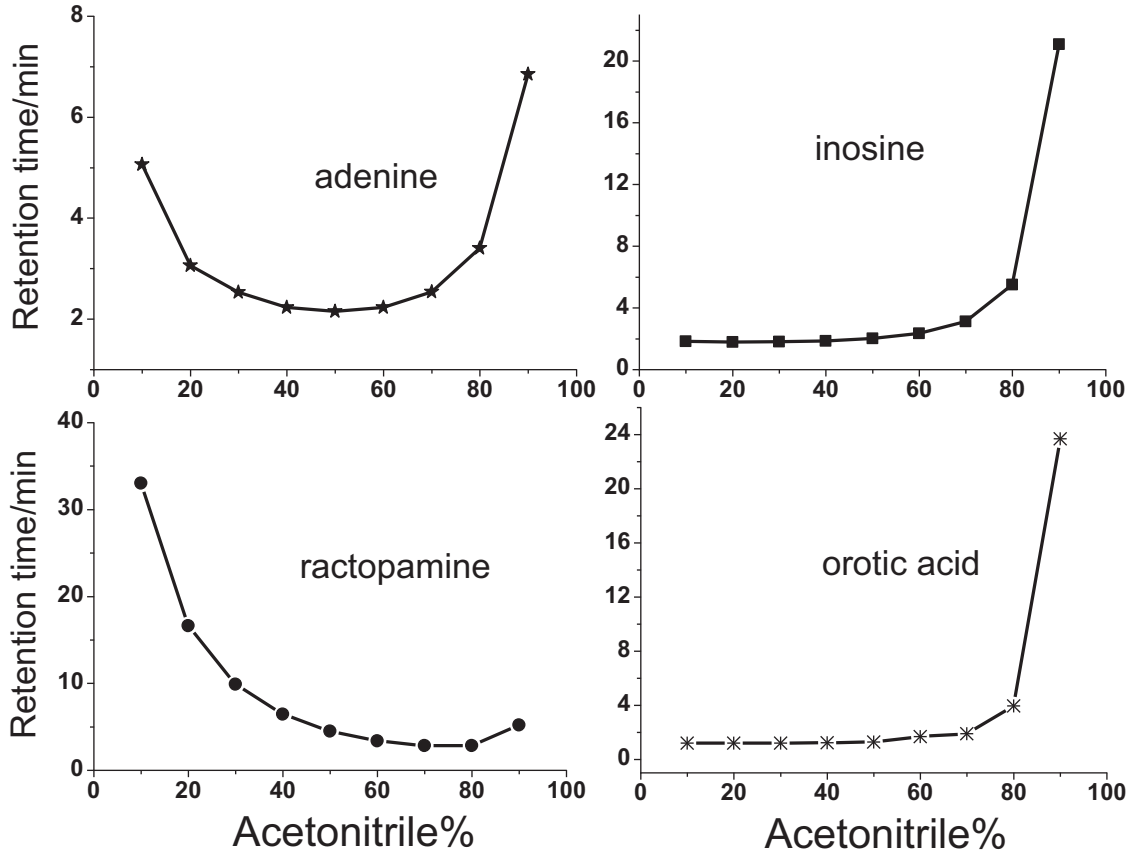

Fig. 4. The influence of CH_3CN content on the retention of small polar compounds on Click TE-GSH column. Experimental conditions: mobile phase, 10 mM ammonium acetate (NH_4Ac, pH 4.9) in CH_3CN/H_2O (CH_3CN content as noted in the figure); flow rate, 1 mL/min; column temperature, 30 °C.

surface. The retention of inosine (pK_a 1.2, 8.9) and orotic acid (pK_a 2.8) decreased obviously with the decrease of CH_3CN content from 90% to 80% in the mobile phase and remained almost unchanged within CH_3CN content of 50% to 10%, i.e., the retention was mainly influenced by the partitioning of solutes between the mobile phase and surface water-enriched layer on Click TE-GSH stationary phase. Besides, the electrostatic repulsion between orotic acid and the negatively charged surface might also affect its retention. The retention of ractopamine (pK_a 9.4) increased when the CH_3CN content was increased from 80% to 90%. But the retention of ractopamine was weak in HILIC mode owing to its hydrophobicity. With the decrease of CH_3CN content from 80% to 10%, the retention of ractopamine increased markedly especially when the CH_3CN content was changed from 20% to 10%. Thus, the retention of ractopamine was influenced predominantly by the electrostatic attraction between the solute and the stationary phase. The results indicated that Click TE-GSH material provided both cation-exchange and hydrophilic interaction during the separation of polar charged compounds.

To further investigate the ion-exchange characteristics on Click TE-GSH stationary phase, the effect of pH values in buffer solution on the retention of basic, neutral and acidic compounds was conducted in HILIC mode. As displayed in Table 1, the retention times of tested compounds remained almost unchanged with the increase of pH from 3.9 to 5.7, i.e., the retention was dominated by hydrophilic partitioning mechanism and influence of electrostatic interaction was minor in HILIC mode. In addition, the influence of pH values on the retention of ionic solutes on Click TE-GSH column in IEX mode was tested (Table 2). The retention of melamine (pK_a 5.0), ractopamine (pK_a 9.4), clenbuterol (pK_a 9.6) and salbutamol (pK_a 9.3) were enhanced with increasing pH value. As pH changed from 3.1 to 4.9, the negative charge on the surface of the stationary phase was increased. Thus, electrostatic attraction between the solutes and Click TE-GSH stationary phase became stronger. In contrast, the retention of salicylic acid (pK_a 3.0) and orotic acid (pK_a 2.8) decreased since the electrostatic repulsion was increased. Meanwhile, the retention of cytidine (pK_a 4.7, 12.2) and adenine (pK_a 4.2, 9.8) was increased from pH 3.1 to 4.0 and then the retention became weak when pH changed from 4.0 to 4.9. It was considered likely that the net charges of the solutes changed within this pH range, affecting their retention.

On the other hand, the hydrophilicity of Click TE-GSH column was compared with commercially available zwitterionic columns (ZIC-HILIC, ZIC-cHILIC) and Silica column (Fig. S2 and Table S1, SI). The results demonstrated that Click TE-GSH column possessed better hydrophilicity.

Table 1
The influence of pH values on the retention times of small polar compounds in HILIC mode. Experimental conditions: mobile phase, 10 mM NH_4Ac (pH as noted) in CH_3CN/H_2O (85:15, v/v); flow rate, 1 mL/min; column temperature, 30 °C.

Compounds (min)	pH		
	3.9	4.8	5.7
Inosine	9.16	9.04	8.98
Cytidine	12.96	12.63	12.36
Adenosine	5.14	5.07	5.00
Adenine	4.50	4.44	4.40
Ractopamine	3.28	3.24	3.27
Propranolol	2.60	2.61	2.68
Orotic acid	8.66	8.19	7.55
Uridine	5.68	5.61	5.54

Table 2
The influence of pH values on the retention of small polar compounds in IEX mode. Experimental conditions: mobile phase, 20 mM NH_4FA (pH as noted) in CH_3CN/H_2O (10:90, v/v); flow rate, 1 mL/min; column temperature, 30 °C.

Compounds	pH		
	3.1	4.0	4.9
Salicylic acid	2.42	1.53	1.28
Orotic acid	1.75	1.34	1.24
Melamine	3.37	8.83	12.85
Cytidine	2.59	3.95	3.12
Adenine	3.28	5.45	4.45
Ractopamine	3.03	7.85	18.16
Clenbuterol	4.53	14.67	39.66
Salbutamol	2.53	5.90	13.03

3.3. Versatility and applicability of Click TE-GSH stationary phase

3.3.1. Application in the separation of neutral, basic and acidic oligosaccharides

Carbohydrates are gaining more and more attention due to their biological functions [34–36]. The separation of carbohydrates is a great challenge as they are poorly retained by reversed-phase liquid chromatography (RPLC). In consideration of the appreciable hydrophilicity of Click TE-GSH zwitterionic material, the separation of fructosan in HILIC mode was studied. As shown in Fig. 5, fructosan with degree of polymerizations (DPs) ranging from 2 to 48 were separated with good peak shape. The higher the DP of a fructooligosaccharide was, the more it associated with the water-enriched layer on the stationary phase and the later it eluted. Taking advantage of the cation-exchange characteristics of Click TE-GSH column, the resolution of chitooligosaccharides in CEX mode and HILIC/CEX mixed-mode was also performed. Chitooligosaccharides, which are positively charged at pH 3.0, could be effectively retained by electrostatic attraction with the carboxyl groups on the stationary phase. With increasing DP of chitooligosaccharides, there are more positively charged amino groups to interact with the negatively charged surface. Therefore, the retention time of chitooligosaccharides with higher DP was much longer (Fig. 6a). By contrast, chitooligosaccharides cannot be retained on a Silica column under the same condition (Fig. S3, SI). In addition, the separation of chitooligosaccharides in HILIC/CEX mixed-mode was also conducted (Fig. 6b). The introduction of hydrophilic interaction with the addition of organic solvent in the mobile phase greatly increased the retention of chitooligosaccharides.

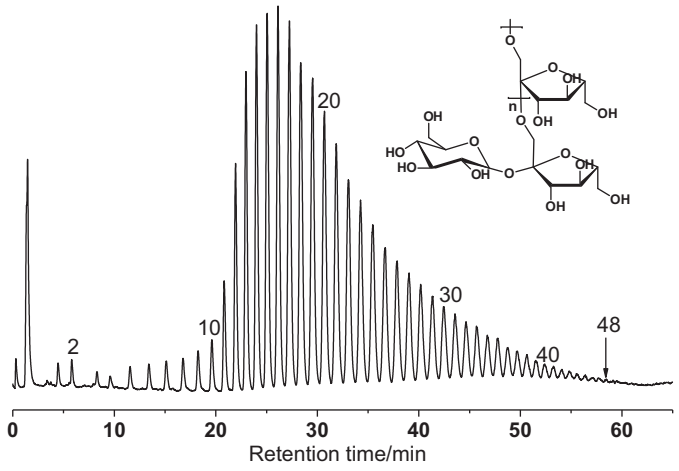

Fig. 5. The separation of fructosan on Click TE-GSH column. Experimental conditions: mobile phase A, H_2O; B, CH_3CN; gradient: 0–20 min, 30–50% A, 20–60 min, 50–60% A, 60–70 min, 60–70% A; flow rate, 1 mL/min; column temperature, 30 °C; ELS detector: gas pressure 30 psi, tube temperature 80 °C, gain 10.

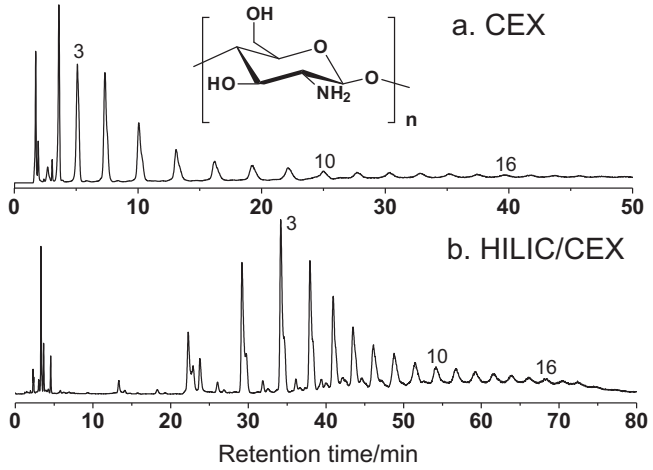

Fig. 6. The separation of chitooligosaccharides on Click TE-GSH column in (a) CEX mode and (b) HILIC/CEX mode. Experimental conditions: mobile phase A, CH_3CN; B, 200 mmol/L ammonium formate (NH_4FA, pH 2.9); C, H_2O; gradient: (a), 0–50 min, B/C (10:90, v/v) to B/C (100/0, v/v); (b), 0–40 min, A/B (60/40, v/v) to A/B (20/80, v/v), 40–70 min, A/B (20/80, v/v) to A/B (5/95, v/v), 70–80 min, A/B (5/95, v/v) to A/B (5/95, v/v); flow rate, 1 mL/min; column temperature, 30 °C; ELS detector: gas pressure 30 psi, tube temperature 85 °C, gain 100.

The separation of chitooligosaccharides in CEX and HILIC/CEX mode without the need for excessively high content of organic solvent promotes solubility, which is of great importance for further preparative separation. In addition, the separation of strongly acidic carrageenan oligosaccharides was also evaluated on the HILIC/CEX stationary phase. As shown in Fig. 7a, electrostatic repulsion between their sulfate groups and the negatively charged surface resulted in the elution of carrageenan oligosaccharides before the void volume (t_0 1.75 min) in ion-exchange chromatography (IEX) mode. With the combination of hydrophilic interaction, i.e., electrostatic repulsion hydrophilic interaction chromatography [37] (ERLIC), the separation was successfully achieved with good selectivity (Fig. 7b). Presumably the doublet peaks associated with each oligosaccharide correspond to the alpha- and beta-anomers at the reducing end. It should be noted that the application of ERLIC for resolving strongly acidic oligosaccharides could effectively avoid the utilization of high concentrations of buffer solution as in Fig. 6. The results demonstrated that the resolution of

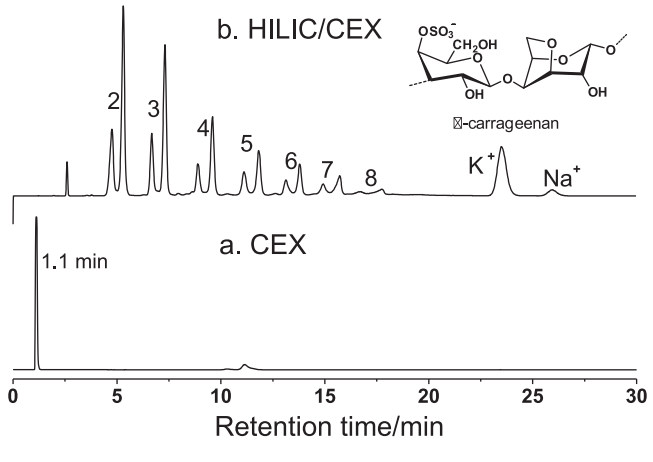

Fig. 7. The separation of carrageenan oligosaccharides on Click TE-GSH column in (a) CEX mode and (b) HILIC/CEX mode. Experimental conditions: mobile phase A, CH_3CN; B, 100 mmol/L NH_4FA (pH 4.2); C, H_2O; gradient: (a), 10% B, 90% C (b), 0–30 min, 75–65% A, with 10% B; flow rate, 1 mL/min; column temperature, 30 °C; ELS detector: gas pressure 30 psi, tube temperature 70 °C, gain 100.

Table 3
Retention of peptide standards in CEX and HILIC/CEX mode.

Peptides	Peptide sequence	Retention times in HILIC/CEX mode	Retention times in CEX mode	No. of potentially positively charged residues	No. of potentially negatively charged residues	No. of polar residues	No. of nonpolar residues	GRAVY[a]	Mw[b]	p*I*[b]
CT127	KRQYKSILQEENRR	19.50	15.57	6	3	12	2	−2.329	1848.10	10.27
CT128	NRAESFRQLWDGARK	14.22	9.94	5	3	9	6	−1.553	1834.03	10.74
CT130	GFGRYRRHGSPW	13.49	15.18	5	1	6	6	−1.642	1475.64	11.71
CT133	DAEFRHDSGYE	11.60	3.51	3	5	8	3	−1.782	1325.31	4.31
CT135	FAGLRQAVTQGFPTEL	5.74	2.77	2	2	6	10	0.138	1734.07	6.00
GFP	EGVNDNEEGFFSAR	8.34	2.45	2	5	8	6	−1.107	1570.68	4.00

[a] GRAVY (grand average of hydropathicity) was estimated on http://www.expasy.org with ProtParam. It was tenable when the amino-acid residues in the peptides were in un-ionized form.
[b] Mass and p*I* were estimated on http://www.expasy.org with Compute p*I*/MW.

neutral oligosaccharides, basic oligosaccharides and strong acidic oligosaccharides could be facilely realized on Click TE-GSH column.

3.3.2. Application in the resolution of model peptides

Considering the existence of both hydrophilic interaction and cation-exchange characteristics on Click TE-GSH stationary phase, the separation of model peptides varying in both hydrophobicity/hydrophilicity and charge (Table 3) was carried out in CEX and HILIC/CEX modes for understanding the interaction mechanism. In CEX mode (pH 3.0), the amino groups of peptides were presumably ionized and carboxylic groups were un-ionized. CT127 peptide which possessed a net charge of +6 was the most retained peptide. Peptide CT130 with a net charge of +5 was almost co-eluted with CT127, which might be caused by the stronger hydrophobic interaction between CT130 and Click TE-GSH stationary phase. Peptide CT128 with more polar residues (weaker hydrophobic interaction) and a net charge of +5 was eluted earlier than CT130. Peptide CT133 which possessed a net charge of +3 was eluted before CT128. And peptide CT135 with the same charge (+2) as peptide GFP but more nonpolar residues was eluted after GFP (representative chromatogram was displayed in Fig. S4b, SI). In consequence, the elution of peptides on Click TE-GSH column in CEX mode was influenced by the electrostatic attraction and hydrophobic interaction. In addition, the separation of model peptides was conducted in the HILIC/CEX mode at pH 3.0. Considering the high content of acetonitrile in the mobile phase, sodium perchlorate ($NaClO_4$) was used as eluting salt due to its good solubility in organic solvent. Peptide CT127 which possessed the most polar residues (highest hydrophilicity) and a net charge of +6 was retained the most (Table 3). Peptide CT128 with lower hydrophilicity and a net charge of +5 was eluted before peptide CT127. The retention of peptide CT130 which had a net charge of +5 but less polar residues was weaker than peptide CT128. CT135 with the highest hydrophobicity and a net charge of +2 was eluted first. GFP with less hydrophobicity but the same charge of +2 was eluted after CT135, while peptide CT133 with higher hydrophilicity and a net charge of +3 was eluted after GFP (Fig. S4a, SI). As shown in Fig. 8, the separation of six highly charged peptides on Click TE-GSH column in CEX mode and HILIC/CEX mixed-mode could be both achieved with high separation efficiency. The resolution exhibited different selectivity for CEX and HILIC/CEX modes.

3.3.3. Application in the separation of a human serum albumin (HSA) tryptic digest

To further demonstrate the applicability of Click TE-GSH stationary phase for complex samples, the separation of a HSA tryptic digest in the HILIC/CEX mode was investigated. The HSA tryptic digest was first desalted with C18 material and redissolved with initial mobile phase. Then the separation of the peptides was performed within 60 min. As shown in Fig. 9, Click TE-GSH stationary

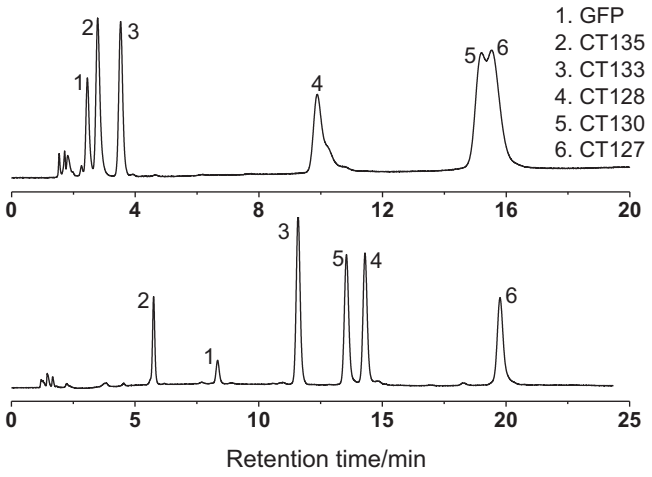

Fig. 8. The separation of standard peptides in (a) CEX and (b) HILIC/CEX mode. Experimental conditions: (a) mobile phase A, CH_3CN; B, 200 mmol/L sodium dihydrogen phosphate (NaH_2PO_4, pH 3.0); C, H_2O; gradient: 0–20 min 5–50% B, 75–30% C, with 20% A; (b) mobile phase A, CH_3CN; B, 500 mmol/L $NaClO_4$; C, 20 mmol/L NaH_2PO_4 (pH 3.0); gradient: 0–20 min 70–40% A, 0–30% B, with C of 30%; flow rate, 1 mL/min; column temperature, 30 °C; detection wavelength, 210 nm.

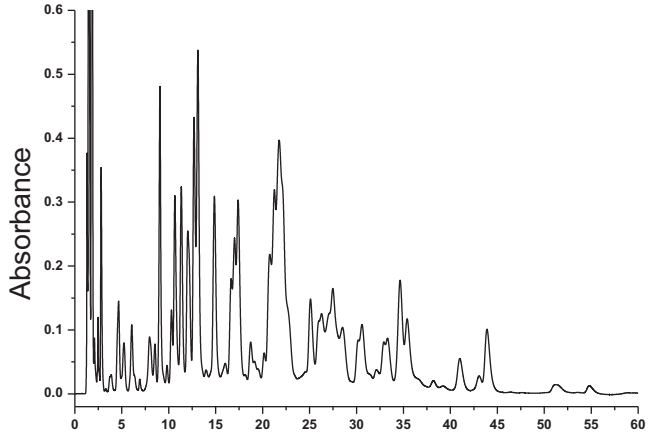

Fig. 9. The separation of a HSA tryptic digest in HILIC/CEX mode. Inset shows the enlarged figure. Experimental conditions: mobile phase A, CH_3CN; B, 500 mmol/L $NaClO_4$; C, 20 mmol/L NaH_2PO_4 (pH 3.0); gradient: 0–55 min, A/B/C (70:0:30, v/v/v) to A/B/C (50:20:30, v/v/v), 55–60 min, A/B/C (50:20:30, v/v/v) to A/B/C (40:30:30, v/v/v); flow rate, 1 mL/min; column temperature, 30 °C; detection wavelength, 210 nm.

phase showed good peak shape and separation efficiency. The eluate was collected every 2 min manually, dried by lyophilization and then redissolved in H_2O/FA (100:0.1, v/v). After desalting, the peptide fractions were directly infused to nano-ESI-MS and characterized. Up to 86 HSA peptides with different mass and charges were matched when comparing to the mass and charges of the theoretical tryptic peptides (Table S2, SI) and sequence coverage of 85% was achieved, demonstrating the potential of Click TE-GSH stationary phase in the resolution of complex peptide mixtures.

4. Conclusion

A novel glutathione-based HILIC/CEX mixed-mode stationary phase (Click TE-GSH) with adjustable surface charge density was facilely synthesized via copper-free "thiol-ene" click chemistry. The investigation of chromatographic retention behaviors on Click TE-GSH column proved that the zwitterionic material possessed good hydrophilicity and inherent cation-exchange characteristics. Fructosan with DP between 2 and 48 was well resolved with high efficiency in HILIC mode. The separation of basic chitooligosaccharides was also achieved both in HILIC/CEX and CEX mode, which is favorable for its preparative separation benefits from lower content of organic solvent. In addition, strongly acidic carrageenan oligosaccharides were successfully separated in the ERLIC mode with low content of volatile buffer. Click TE-GSH stationary phase displayed good efficiency in the separation of highly charged peptides in HILIC/CEX and CEX modes. Peptides varying in both hydrophobicity/hydrophilicity and charge were separated with different selectivity. Finally, the resolution of HSA peptides in the HILIC/CEX mode showed good peak shape and efficiency. In summary, the combination of two widely different separation mechanisms on Click TE-GSH material, i.e., ion-exchange interaction overlaid on hydrophilic interaction exhibits good potential for the resolution of complex samples in glycomics and proteomics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.09.002.

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