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Click novel glycosyl amino acid hydrophilic interaction chromatography stationary phase and its application in enrichment of glycopeptides

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ABSTRACT

A novel glycosyl amino acid hydrophilic interaction chromatography (HILIC) stationary phase was prepared *via* click chemistry. The key intermediate N_3-glycosyl D-phenylglycine was prepared by a three steps procedure, including selective condensation of amino glucose with N-succinimidyl ester of Boc-D-phenylglycine, deprotection and transformation of amino group to azido group. The structure of all the intermediates and functionalized silica beads were confirmed by ^{1}H NMR, IR, elemental analysis and ^{13}C CP-MAS. The chromatography test showed that this new type of separation material possessed good HILIC properties and glycopeptide enrichment characteristics. Nucleosides and bases could be separated in a simple eluent composition (only acetonitrile in combined with water), and with the same condition, these model compounds could not be separated on the commercial HILIC column (Atlantis). Click glycosyl amino acid thus prepared also showed longer retention and better separation ability in the separation of polar organic acids.

1. Introduction

Enrichment of glycopeptides played a very important role in the glycoproteomic study of glycoproteins and direct analysis of glycopeptides by mass spectrometry (MS) and tandem MS (MS/MS) [1–12]. The proteolytic cleavage of glycoproteins generally produces not only glycopeptides but also other products such as glycans and nonglycosylated peptides. The glycopeptides usually have relatively low abundance in the peptide mixture in comparison with nonglycosylated peptides and glycans [1,3,13–15]. Furthermore, the existence of nonglycosylated peptides made it difficult to identify the low-abundance glycoproteins and their glycosylated sites without the step of glycopeptide enrichment from biological samples, for the mass spectrometric response glycopeptides were significantly suppressed by nonglycosylated peptides [1,16,17]. All these issues mentioned above decreased the detection sensitivity and complicated the structural analysis of targeted glycoproteins. Therefore, the effective enrichment of glycopeptides prior to MS analysis of complex peptide samples may decrease sample complexity and improve the detection sensitivity.

So far, several types of methods or materials were used for the enrichment of glycopeptides and glycoproteins, such as lectin affinity chromatography [18–27]; boronic acid functionalized composite nanoparticles or mesoporous silica [28,29]; resin with a covalently bound neutral, zwitterionic sulfobetaine functional group for so-called zwitterion chromatography–hydrophilic interaction chromatography (ZIC–HILIC) [4,13,14,30–34]; click maltose [1], click chitooligosaccharide [35] and click OEG-CD matrix [36]. All these methods or materials showed good characteristics for enrichment of glycopeptides. Even so, not all these methods or materials could meet all the requirements for enrichment of glycopeptides due to their different properties and the sources of diversity biological sample. For example, lectin affinity chromatography showed narrow specificity, and it is difficult to capture glycopeptides of different types unless the mixed or sequential mode was used [1,2,16,17]. Furthermore, the glycosyl amino acids may produce good interactions with glycopeptides due to the structure similarities between glycosyl amino acid and glycopeptides. Therefore, these materials covalent bonded with the glycosyl amino acid may produce good interaction with glycopeptides.

On the other hand, click Cu(I) catalyzed azide-alkyne cycloaddition (CuAAC) chemistry has been proved to be a powerful strategy due to its several advantages: first, this transformation are easy to perform and give intended products with high yield with little or no byproducts; second, organic azides and terminal alkynes are inert to most chemical functionalities and stable to wide range of solvent, temperature and pH; finally, the 1,2,3-triazole moiety has aromatic character, a dipole moment approximately equal to that of an amide bond and the ability to participate in hydrogen bonding [37,38]. Up to now, several type of organic function groups were covalent bonded to silica beads or polymers successfully and several type liquid chromatography stationary phases were prepared and applied in different purpose [35,39–51]. Continuing on research interest on finding new hydrophilic chromatography packings, a novel glycosyl

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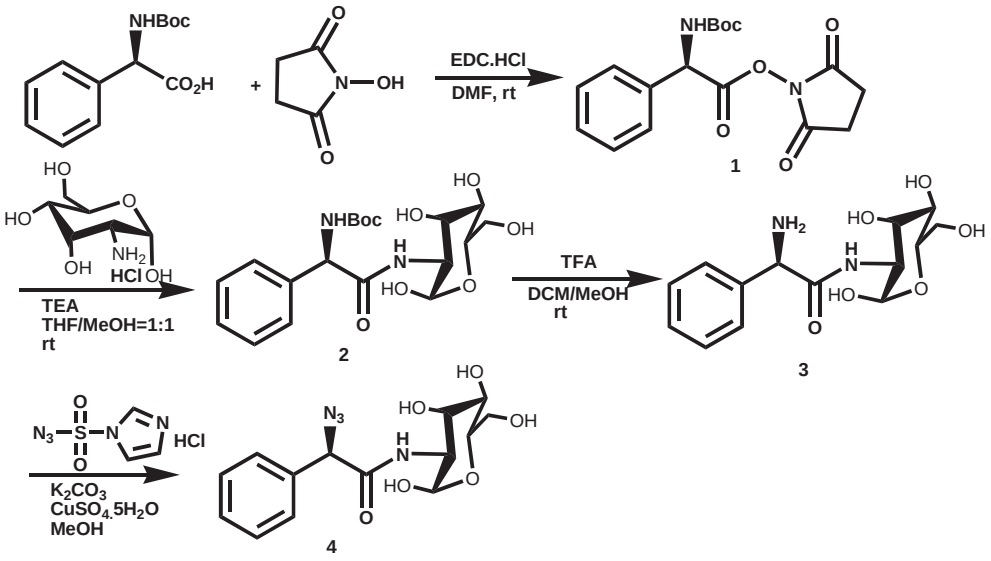

Fig. 1. Preparation of azido glycosyl phenyl glycine.

amino acid (glycosyl phenyl glycine) hydrophilic interaction chromatography (HILIC) stationary phase was designed and the HILIC and glycopeptides enrichment characteristics were expected.

2. Experimental

2.1. Chemicals and reagents

Spherical silica (5 μm particle size; 10 nm pore size; 300 $m^2\ g^{-1}$ surface area) was purchased from Fuji Silysia Chemical Ltd. (Japan). HPLC grade acetonitrile and formic acid were purchased from Tedia (USA) and Acros (USA), respectively. Water was purified on a Milli-Q system (USA). 3-Isocyanatopropyl-triethoxysilane and propargylamine were domestic reagents and purified by distillation before use. Aminoglucose hydrochlorate and Boc-phenylglycine were purchased from Aladdin. Nucleosides and bases were purchased from Sigma, Acros. IgG was obtained from Sigma. Sequencing-grade-modified trypsin was purchased from Promega. GELoader tips were obtained from Eppendorf.

2.2. Synthesis of glycosyl amino acid [52–54]

Starting from Boc-D-phenylglycine and aminoglucose, the key intermediate N_3-glycosyl D-phenylglycine was prepared by several steps (Fig. 1). N-succinimidyl ester of Boc-D-phenylglycine (**1**): a mixture of Boc-D-phenylglycine (12.56 g, 50 mmol), N-hydroxyl succinimide (6.33 g, 55 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) (10.54 g, 55 mmol) in 20 mL anhydrous dimethylformamide (DMF) was stirred at room temperature for 18 h. After reaction, 200 mL of ethyl acetate was added and the mixture was washed well with H_2O, $NaHCO_3$ and saturated brine. The organic layer was dried over anhydrous sodium sulfate. Removed all of volatiles afforded a crude solid, which was recrystallized from ethanol to give a white solid (6.3 g, 18.1 mmol, yield 36%), ^{1}H NMR ($CDCl_3$) δ 7.26–7.46 (m, 5H), 5.74 (d, 1H, J = 8 Hz), 5.38 (br, 1H), 2.81 (s, 4H), 1.46 (s, 9H); N-glycosyl-Boc-D-phenyl glycine (**2**): a stirred suspension of N-succinimidyl ester of Boc-D-phenylglycine (**1**) (6.3 g, 18 mmol) and aminoglucose (2.59 g, 12 mmol) in anhydrous MeOH (60 mL) and THF (40 mL) was cooled to 0 °C, then triethylamine (1.28 g, 12.6 mmol) was added under nitrogen atmosphere. The mixture was stirred at room temperature for about 29 h, and the completion of the reaction was detected by thin layer chromatography (TLC). After reaction, all solvent was removed by evaporation to afford a residue, which was purified by a silica gel column (ethyl acetate:MeOH = 10:1 as eluent) to get the target compound **2** (3.28 g, 7.9 mmol, yield 44%). ^{1}H NMR (CD_3COCD_3) δ: 7.26–7.51 (m, 5H), 6.46 (br, 1H), 5.73, 5.64 (anomers, 1H), 5.35 (d, J = 6.8HZ, 1H), 5.20 (br, 1H), 3.63–3.92 (m, 6H), 1.40 (s, 9H). The removal of Boc group in N-glycosyl-Boc-D-phenyl glycine (**2**): compound 2 (3.28 g, 7.9 mmol) was dissolved in a mixture of methanol and CH_2Cl_2 (1:1, v/v), 15 mL triflouro acetic acid was added. The mixture was stirred at rt overnight until no starting material was detected by TLC. Removal of all solvents left 2.31 g yellow solid **3**, which was used directly to next step without further purification; N-glycosyl-N_3-phenyl glycine (**4**): a suspension of N-glycosyl-phenyl glycine (**3**, 2.31 g, 7.3 mmol), $CuSO_4{\cdot}5H_2O$ (18.5 mg, 0.07 mmol), K_2CO_3 (2.29 g, 16.6 mmol) and imidazole-1-sulfonyl azide hydrochloride (1.84 g, 8.8 mmol) in MeOH (40 mL) was stirred at room temperature for 24 h. The pH was adjusted to 1 by adding concentrated HCl (12 N). Removal of all solvents left a residue, which was purified on silica gel column to get a pale yellow solid (N_3-glycosyl phenyl glycine **4**). ^{1}H NMR (CD_3COCD_3) δ 7.35–7.47 (m, 5H), 5.36 (br, 1H), 5.23, 5.18 (anomers, 1H), 3.85–3.46 (7H); IR (cm^{-1}) 3300, 2115, 1645, 950.

2.3. Preparation of click glycosyl phenyl glycine

The alkynyl-modified silica gel (**5**) was synthesized according to reported method (Fig. 2) [35,39]: to the solution of 3-isocyanatopropyltriethoxysilane (35.0 mmol, 8.65 g) in 100 mL anhydrous DMF was added propargylamine (42.0 mmol, 2.31 g). The mixture was stirred at 85 °C for 12 h. Cooled to room temperature. Thus prepared solution was then transferred to a flask containing 10 g silica gel. The suspension was stirred for another 32 h at 110 °C and filtered. After washed with 300 mL dichloromethane, 500 mL methanol and 250 mL acetone, the solid was collected by filtration and dried in high vacuum. The product was confirmed by FT-IR spectra and elemental analysis. Elemental analysis (%): C 8.04; N 2.46; H 1.39. And the surface coverage of the alkynyl-modified was calculated to be 2.82 μmol/m^2.

The N_3-glycosyl phenyl glycine was covalent bonded to silica beads by click strategy (Fig. 2.): to a solution of N_3-glycosyl phenyl glycine in H_2O/MeOH (30 mL/60 mL) was added sodium ascorbate

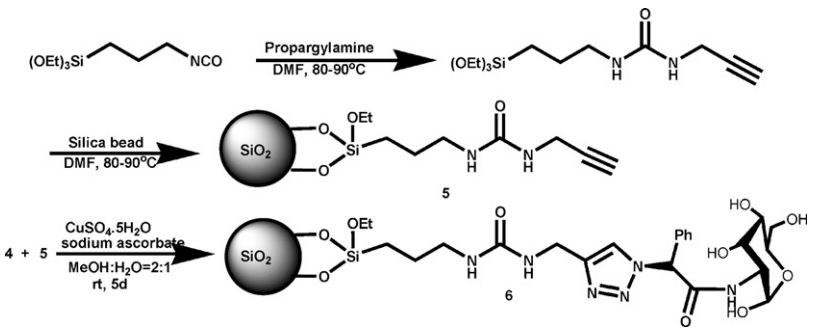

Fig. 2. Preparation of click glycosyl phenyl glycine on silica beads.

(0.19 mmol, 50 mg) (dissolved in 2 mL H_2O), $CuSO_4$ (0.56 mmol, 25 mg) (dissolved in 2 mL H_2O) and alkynyl-modified silica (2.5 g) subsequently. After stirring at room temperature for 5 days, the mixture was filtered and washed with hot 10% ethylenediaminetetraacetic acid (EDTA) (200 mL), hot water (300 mL), hot methanol (150 mL 3×) and acetone (300 mL). After dried in high vacuum, the product click glycosyl phenyl glycine (**6**) was characterized by elemental analysis, FT-IR spectra (cm^{-1}) and ^{13}C CP-MAS (Fig. 3). Elemental analyses (%): C 12.48; N 3.91; H 2.02. And the surface coverage of the click glycosyl phenyl glycine based on carbon increase was calculated to be 0.88 μmol/m^2.

2.4. Apparatus and chromatography

The click glycosyl phenyl glycine stationary phase was dispersed into methanol and packed into stainless-steel column (100 mm × 2.1 mm I.D.) by a slurry packing technique for evaluation of the click glycosyl phenyl glycine stationary phase.

The effect of acetonitrile content in mobile phase, buffer salt and pH value, as well as column efficiency evaluation of the click glycosyl phenyl glycine stationary phase were performed on Agilent HPLC system, which is comprised of an Agilent 1200 series. The chromatographic experiments were carried out at 30 °C. The UV absorbance detection was performed at 254 nm and the flow rate of the mobile phase was 0.2 mL/min.

Enrichment of glycopeptides conditions: an inert sieve was placed in the end of the GELoader tip prior to packing. For microcolumn packing, acetonitrile slurry (45 μL) containing click glycosyl phenyl glycine was pushed into the GELoader tip. The resulting microcolumn was first washed with a $CH_3CN/H_2O/HCO_2H$ solvent mixture (10:90:0.1 (v/v/v), 45 μL) and equilibrated with $CH_3CN/H_2O/HCO_2H$ (80:20:0.1 (v/v/v), 90 μL). Then tryptic digest (30 μL) was dried, re-dissolved in $CH_3CN/H_2O/HCO_2H$ (80:20:0.1 (v/v), 30 μL), and loaded onto the column. The column was rinsed with $CH_3CN/H_2O/HCO_2H$ (80:20:0.1, v/v/v), 30 μL) for three times to remove non-glycopeptides. The glycopeptide fraction (human IG digest) was eluted with $CH_3CN/H_2O/HCO_2H$ (70:30:0.1, 60:40:0.1, v/v/v respectively), collected, dried and analyzed by MS.

3. Results and discussion

The click glycosyl phenyl glycine stationary phase was analyzed by FT-IR spectra and element analysis. The IR spectrum of click glycosyl phenyl glycine stationary phase was similar to alkynyl-silica, which may be contributed to the low surface concentration of alkynyl group and its weak absorption. In ^{13}C CP-MAS NMR (Fig. 3), signals around 164 ppm were attributed to carbonyl carbon atoms (g and l) and these signals between 152 ppm and 129 ppm were ascribed to the aromatic carbons such as phenyl and 1,2,3-triazole ring (i and j). The signal at 91 ppm was assigned to the anomeric carbon (a). These signals between 65 ppm and 50 ppm were due to the chiral carbon (h) on phenyl glycine and these carbons on sugar (b, c, d, e, f). These signals around 45 ppm were ascribed to these carbons connected to nitrogen atom (m and k), and signals around 24 ppm and 9 ppm were attributed to alkyl carbon chain (n) and these carbons connected to silica atoms (o) respectively. The elemental analysis data showed that the carbon content increased from 8.04% (alkynyl-silica) to 12.48% (click glycosyl phenyl glycine stationary phase). All of these facts demonstrated that the gycosyl phenyl glycine was successfully bonded to the surface of silica. And the surface concentration of click glycosyl phenyl glycine stationary phase was 0.88 μmol/m^2 based on the increase of carbon content.

It is worth notice that the only one reaction site on N_3-glycosyl phenyl glycine makes the click glycosyl phenyl glycine stationary phase highly order, it may afford special performance on HILIC and enrichment of glycopeptides.

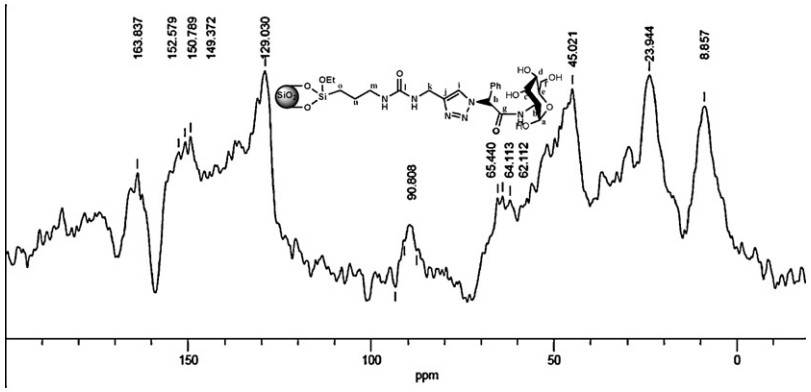

Fig. 3. ^{13}C CP-MAS of click glycosyl phenyl glycine stationary phase.

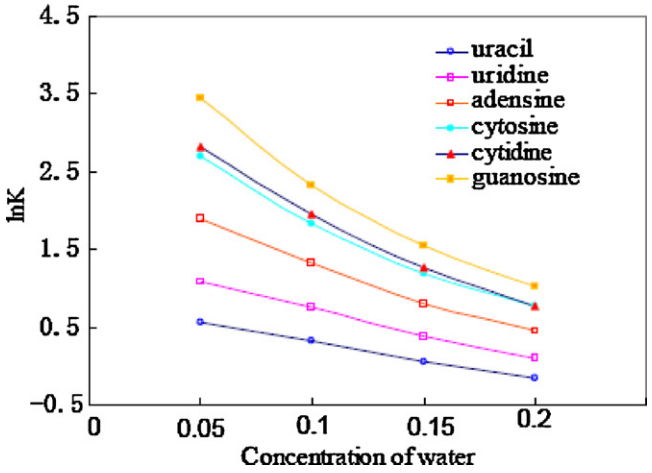

Fig. 4. Effect of water content in the mobile phase on retention. Mobile phase: ACN/water, flow rate 0.2 mL/min, UV detection: 254 nm.

The effects of water concentration in mobile phase and buffer salt concentration on chromatographic characteristic showed that all these properties were typical HILIC characteristics. For example, the retention time of nucleosides and bases became longer with the increasing of the concentration of acetonitrile in mobile phase (Fig. 4). While the higher buffer salt concentration decreased the retention time of solutes (Fig. 5).

The chromatographic evaluation of the click glycosyl phenyl glycine stationary phase was performed in acetonitrile/water eluents. Under this condition, the nucleosides and bases could be well separated on click glycosyl phenyl glycine stationary phase and click chitooligosaccharide, while these model compounds could not been separated on commercial Atlantis HILIC-column (Fig. 6). It is worth noticing that these kind model samples can be separated on click glycosyl phenyl glycine with simple eluent without any butter. Usually the buffer was used in HILIC model in order to obtain better chromatographic characteristics. The chromatograms are shown in Fig. 6a. Obviously, this stationary phase exhibited good interactions to these higher polar compounds, which was similar with click chitooligosaccharide.

This stationary was subsequent applied to the separation of high polar organic acids using 90% ACN, 50 mM HCO_2NH_4 (pH = 4.5) as eluent, and seven high polar organic acids was used as model compounds. These organic acids were well separated on click glycosyl phenyl glycine (Fig. 7).

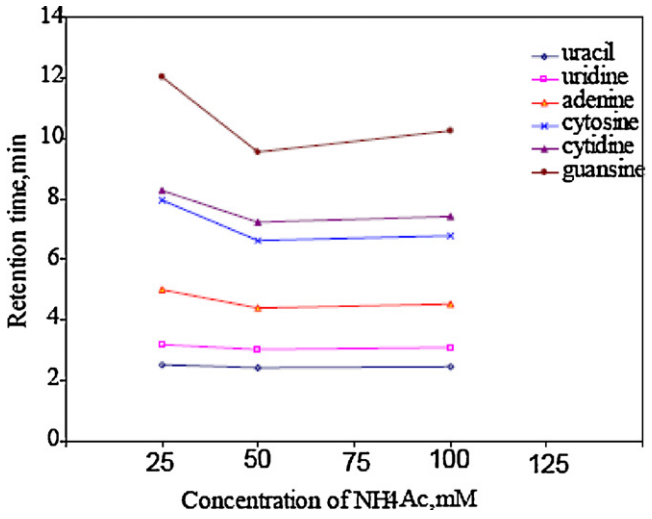

Fig. 5. Effect of salt concentration in the mobile phase on retention. Mobile phase: ACN/NH_4Ac, flow rate 0.2 mL/min, UV detection: 254 nm.

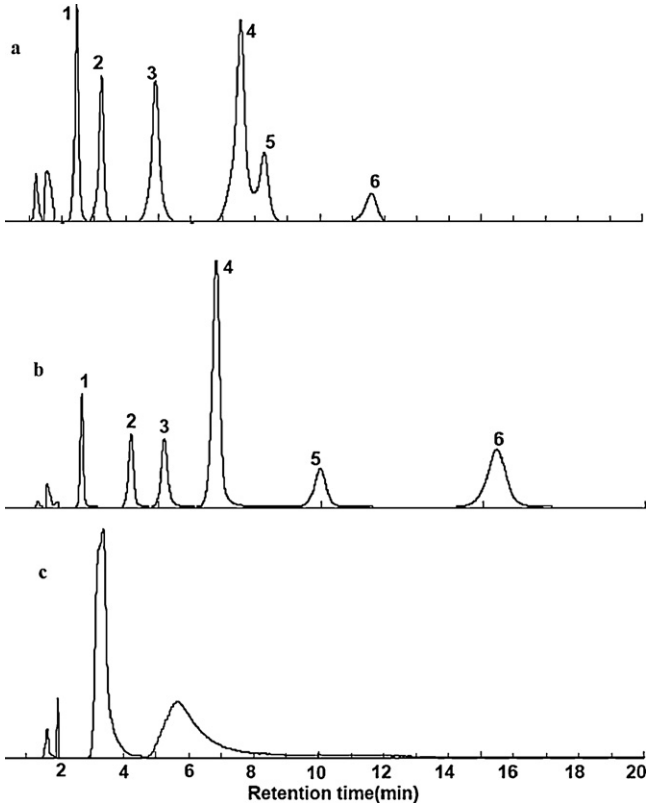

Fig. 6. Separation of nucleosides and bases on click glycosyl phenyl glycine ((a) 5 μm, 2.1 mm × 100 mm), click chitooligosaccharide ((b) 5 μm, 2.1 mm × 100 mm) and Atlantis HILIC-silica ((c) 5 μm, 2.1 mm × 100 mm). Conditions: flow rate 0.2 mL/min; 30 °C; mobile phase: ACN/H_2O = 90:10, UV: 254 nm. Peak identification: 1 uracil, 2 uridine, 3 adenine, 4 cytosine, 5 cytidine, 6 guanosine.

Consider of the hydrophilic characteristics of the click glycosyl phenyl glycine stationary phase and its similar structure to glycopeptides, this type of new material was applied to glycopeptide enrichment and the human IG digest was used as representative sample. As shown in Fig. 8, after click glycosyl phenyl glycine enrichment, the interfering non-glycopeptides were effectively removed and the signal intensities of the glycopeptides were significantly enhanced with a relative high water content solvent ($CH_3CN/H_2O/HCO_2H$ (70:30:0.1, v/v/v), Fig. 8b), especially for those low abundance glycopeptides. And in higher water content solvent

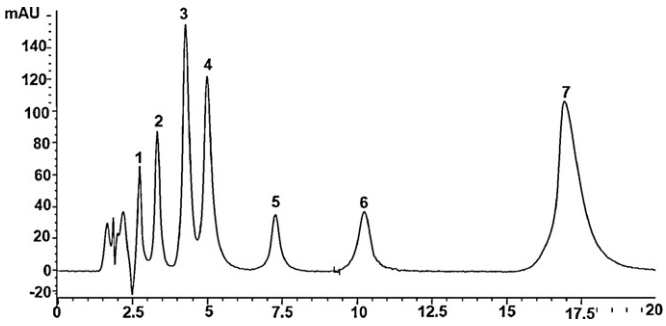

Fig. 7. Separation of organic acids on click glycosyl phenyl glycine ((a) 5 μm, 2.1 mm × 100 mm), and Atlantis HILIC-silica ((b) 5 μm, 2.1 mm × 100 mm). Conditions: flow rate 0.2 mL/min; 30 °C; mobile phase: ACN/50 mM HCO_2NH_4 (pH 4.5) = 90:10, UV: 254 nm. Peak identification: (1) 5-sulfosalicylic acid, (2) N-(2,4-dinitrophenyl)-L-leucine, (3) cinnamic acid, (4) p-aminosalicylic acid, (5) 3,5-dihydroxybenzoic acid, (6) orotic acid, and (7) 2,4-dihydroxybenzoic acid.

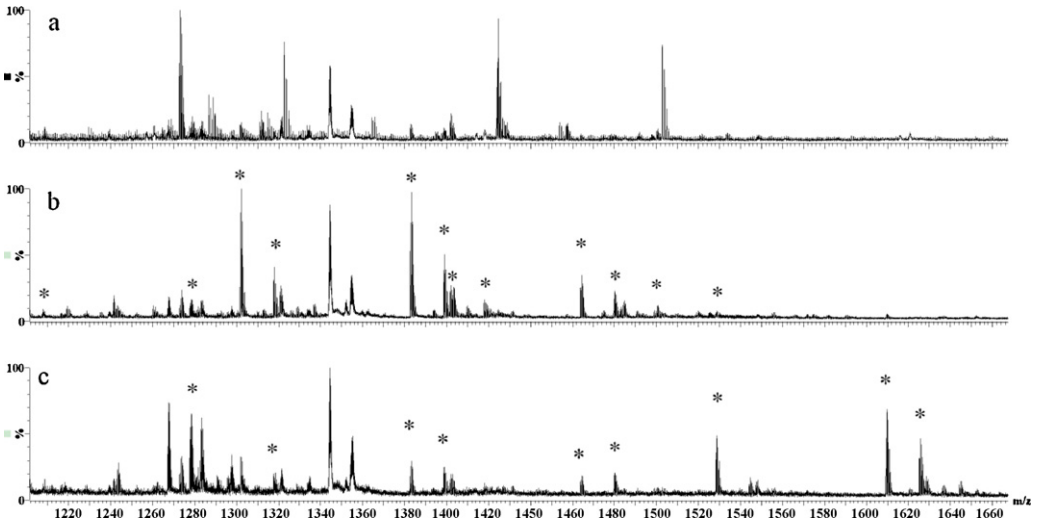

Fig. 8. Nano-ESI-MS of human IgG digest before and after click glycosyl phenyl glycine enrichment. Glycopeptides were marked with asterisk. SPE loading condition: 80% ACN/0.1% FA. (a) Before enrichment; (b) 70% ACN/0.1% FA; (c) 60% ACN/0.1% FA.

($CH_3CN/H_2O/HCO_2H$ (60:40:0.1, v/v/v), Fig. 8c), the larger molecular glycopeptides with sialic acids were retained. Compared with click chitooligosaccharide [35], more glycopeptides were detected after enrichment. Although some non-glycopeptides with more amino acid residuals were co-eluted with the glycopeptides, the click glycosyl phenyl glycine stationary phase still exhibited great selectivity towards glycopeptides.

4. Conclusion

A novel click glycosyl phenyl glycine stationary phase was prepared *via* click chemistry. This material showed typical HILIC characteristics to separate nucleosides and bases, and buffer was not required, which make it much convenient to apply this kind of stationary phase to the Liquid chromatography–mass spectrometry (LC–MS). Based on its hydrophilic characteristics and special interaction raised by similar structures to glycopeptides, it has great potential to enrich glycosylated peptides and other application in the separation of high polar compounds.

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