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SupplementaryInformention for:

Synthesis and evaluation of a silica-bonded concanavalin A material for lectin affinity enrichment of N-linked glycoproteins and glycopeptides

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1. Materials and Methods

Silica gel (particle size 5μm; pore size 300Å; surface area 60 m^2 /g) was purchased from Fuji Silysia Chemical (Kasugai, Japan). Glutaric anhydride, APTES, EDC, NHS, mono-ethanolamine, hydroxyethyl piperazine ethanesulfonic acid (HEPES), and trifluoroacetic acid (TFA) were purchased from J&K Scientific Ltd. Con A, methyl α-D-mannopyranoside, chicken ovalbumin (OVA), RNase B, human serum albumin (HSA), bovine fetuin, dithiothreitol (DTT), iodoacetic acid (IAA), ammonium bicarbonate (NH_4HCO_3), and tetrahydrofuran (THF) were purchased from Sigma (St. Louis, Mo). BCA Protein Assay Reagent (bicinchoninic acid) was purchased from Pierce (Rockford, IL). Trypsin was purchased from Promega (Madison, WI). Tris (hydroxymethyl) aminomethane (Tris), and formic acid (FA) were purchased from Acros (Geel, Belgium). Toluene and N, N-dimethyl formamide (DMF) were purchased from Kemiou Chemical Reagent Co. Ltd. (Tianjin, China). Acetonitrile (CH_3CN, HPLC gradient) was from Merk (Darmstadt, Germany). Water was purified by the Milli-Q system (Milford, MA). Materials for desalting were prepared in our lab. All the solvents were dried with 4 Å sieves before use.

LC-MS analysis was performed on Waters 2998-Masslynx system. Elemental analysis was performed on a Vario EL III system (Elementar, Germany), Fourier transform infrared spectrum (FT-IR) was performed on EQUINOX 55(Oregon, USA), and the content of protein was detected on Bio-tek ELx800 (California, USA).

2. Preparation of SG Con A and packing of the column

Silica gel was dried at 150 ^oC for 8h before the reaction. In the presence of equivalent Et_3N (0.63 μL), 3-aminopropyltriethoxy silane **1** (APTES) (4.5 mmol, 1.1 mL) was added to the solution of the glutaric anhydride **2** (5.4 mmol, 616 mg) in 10 mL anhydrous THF, stirring for 4h at room temperature to generate the silane coupling reagent **3**, then silica gel (2.5 g) in 30 mL dry toluene was added, stirred for 48 h at 80 ^oC to obtain matrix **4**, which was filtered and washed by THF, toluene, methanol, water and methanol successively, then dried at 80 ^oC overnight.

At room temperature, matrix **4** (2.0 g) was suspended in dried DMF (20 mL) with 0.2 M EDC and 0.4 M NHS, the mixture was stirred for 2 h and filtered, followed by washing with DMF and 10 mM HEPES buffer (pH 7.4). At room temperature, the obtained NHS-silica matrix **5** was re-suspended in 20 mM HEPES buffer (10 mL), followed by adding Con A (236 mg) in 50 mL HEPES buffer containing 0.2 M methyl α-D-mannopyranoside as the inhibitor to protect the binding sites. After

stirring gently for 2h, 0.2 M ethanolamine was added to block the reaction for 1 h to obtain the material **6 (**SG Con A**)**, which was washed with HEPES buffer until there was no UV absorption peak being found under 280 nm.

The obtained SG Con A (1.5 g) in HEPES buffer (15 mL) was treated in an ultrasonic bath for 5 min before pouring into stainless steel column (150 mm × 3.0 mm), with 20 mM HEPES buffer as slurry solvent under the pressure of 32 MPa. The column can be kept in 20 mM HEPES buffer containing 0.02% NaN_3 at 4 ^oC for 12 month without affecting its trapping efficiency.

3. Chromatographic conditions

Glycoproteins were separated by SG Con A column, gradient elution that performed on Alliance HPLC system consisted of Waters 2695 pump and Waters 2996 DAD detector (Waters, Milford, MA, USA). The mobile phase A was 10 mM HEPES buffer (pH 7.4, containing 0.15 M sodium chloride, 1 mM calcium chloride, 1mM manganese chloride, 1mM magnesium chloride, 0.02% sodium azide), and the mobile phase B was 0.2 M methyl α-D-mannopyranoside in 10 mM HEPES buffer. The column temperature was held at 20 ^oC at a flow rate of 0.2 mL/min.

A sample of commercial glycoprotein with the concentration of 10 μg/μL, including HSA, ovalbumin, and RNase B, was loaded onto the SG Con A column, respectively, and the fractioned glycoproteins were dialyzed against water to remove the salts.

4. Deglycosylation of N-linked glycoproteins

The release of glycans from the obtained fractions was performed following the previously described [2]. Briefly, the glycoprotein fractions (100 μg) was dissolved in 100 μL 20 mM ammonium bicarbonate (pH 8.5), 0.1 μL PNGase F (500 units per μL) was added to the solution and incubated overnight at 37 ^oC for N-glycan release. After heating to 95 ^oC for 5 min to stop the reaction, the glycans were directly enriched with C18 and Click Maltose sequentially for MS analysis.

5. Enrichment of released glycans from OVA

Firstly, C18 (about 1 mg) was packed into the GE Loader tip with an inert sieve plug up the end, the resulting microcolumn was first equilibrated with $H_2O/CH_3CN/FA$ (80:20:0.1(v/v), 90 μL). Then PNGase F digest (10 μL) was dried, re-dissolved in $H_2O/CH_3CN/FA$ (80:20:0.1 (v/v), 10μL) and loaded onto the column. The column was rinsed with H_2O (90 μL) to remove glycans and salts. The remaining glycoproteins were eluted with $CH_3CN/H_2O/FA$ (50:50:0.1(v/v), 90 μL), dried and

collected.

Secondly, Click Maltose (about 1 mg) was packed into the GE Loader tip with an inert sieve plug up the end, the resulting micro-column was first equilibrated with $CH_3CN/H_2O/FA$ (80:20:0.1(v/v), 90 μL). Then the mixture of glycans was dried, re-dissolved in $CH_3CN/H_2O/FA$ (80:20:0.1 (v/v), 10μL), and loaded onto the column. The column was rinsed with $CH_3CN/H_2O/FA$ (80:20:0.1(v/v), 60 μL) to remove salts. The glycans were eluted with $CH_3CN/H_2O/FA$ (70:30:0.1, 30 μL for two times), collected and dried. The enrichment process was repeated at least three times.

6. Enrichment of RNase B glycopeptides with SG Con A and Agarose Con A

According to the procedure stated in our previous work[3], RNase B glycoproteins were digested with trypsin, enriched with C18 and Click Maltose sequentially for MS analysis[4].

In detail, SG Con A (about 1 mg) was packed into the GE Loader tip with an inert sieve plug up the end, the resulting micro-column was equilibrated with HEPES buffer (pH 7.4) before the tryptic digest (10 μL) was loaded onto the column. Then the column was rinsed with HEPES buffer (pH 7.4, 60 μL) to remove the non-glycopeptides. While the glycopeptide fraction was eluted with 0.2 M methyl α-D-mannopyranoside in 10 mM HEPES buffer (30 μL for two times), collected and dried. The enrichment process was repeated at least three times.

When it turns to Agarose Con A, three replicate enrichment procedures were repeated just by the replacement of material SG Con A.

In order to desalination, C18 (about 1 mg) was packed into the GE Loader tip with an inert sieve plug up the end, the resulting micro-column was first equilibrated with $H_2O/CH_3CN/FA$ (80:20:0.1(v/v), 90 μL). Then tryptic digest (10 μL) was dried, re-dissolved in $H_2O/CH_3CN/FA$ (80:20:0.1 (v/v), 10μL), and loaded onto the column. The column was rinsed with H_2O/CH_3CN /FA (80:20:0.1(v/v), 45 μL) to remove non-glycopeptides as well as salts. The glycopeptide fraction was eluted with $CH_3CN/H_2O/FA$ (60:40:0.1, 30 μL for two times), collected and dried, and three replicate has been performed for enrichment.

Then, Click Maltose (about 1 mg) was packed into the GE Loader tip with an inert sieve plug up the end, the resulting micro-column was first equilibrated with $CH_3CN/H_2O/FA$ (80:20:0.1(v/v), 90 μL). The desalted peptides from C18 were re-dissolved in $CH_3CN/H_2O/FA$ (80:20:0.1 (v/v), 10μL) and loaded onto the column. The column was rinsed with $CH_3CN/H_2O/FA$ (80:20:0.1(v/v), 60 μL) to remove non-

glycopeptides, and eluted with CH_3CN/H_2O/FA (70:30:0.1, 30 μL for two times) to enrich the glycopeptides, and three replicate has been performed for enrichment.

7. Mass Spectrum experiments

MS experiments were performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, Manchester, UK). The nano-ESI source was operated under positive ion mode with nanospray voltage at 2.1 kV. MS data were acquired at m/z 1000-2000 for ovalbumin fractions and 600-2000 for RNase B fractions.

Table S1

Stationary phase	C%	N%	Surface coverage ($\mu mol/m^2$)
Silica particles **4**	6.1	0.7	8.1
Silica particles **5**	7.4	1.1	6.6

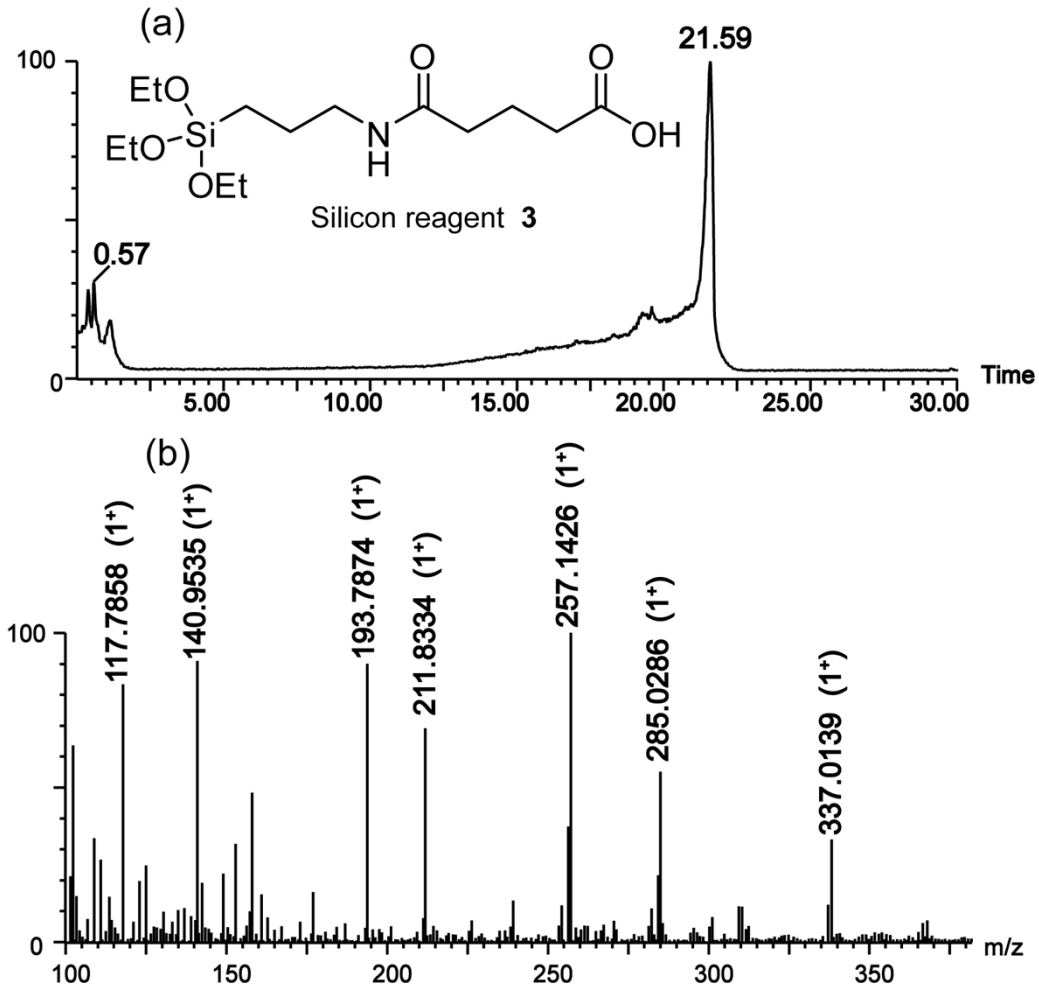

Fig. S1 LC-MS analysis of the synthesized silane reagent **3**.

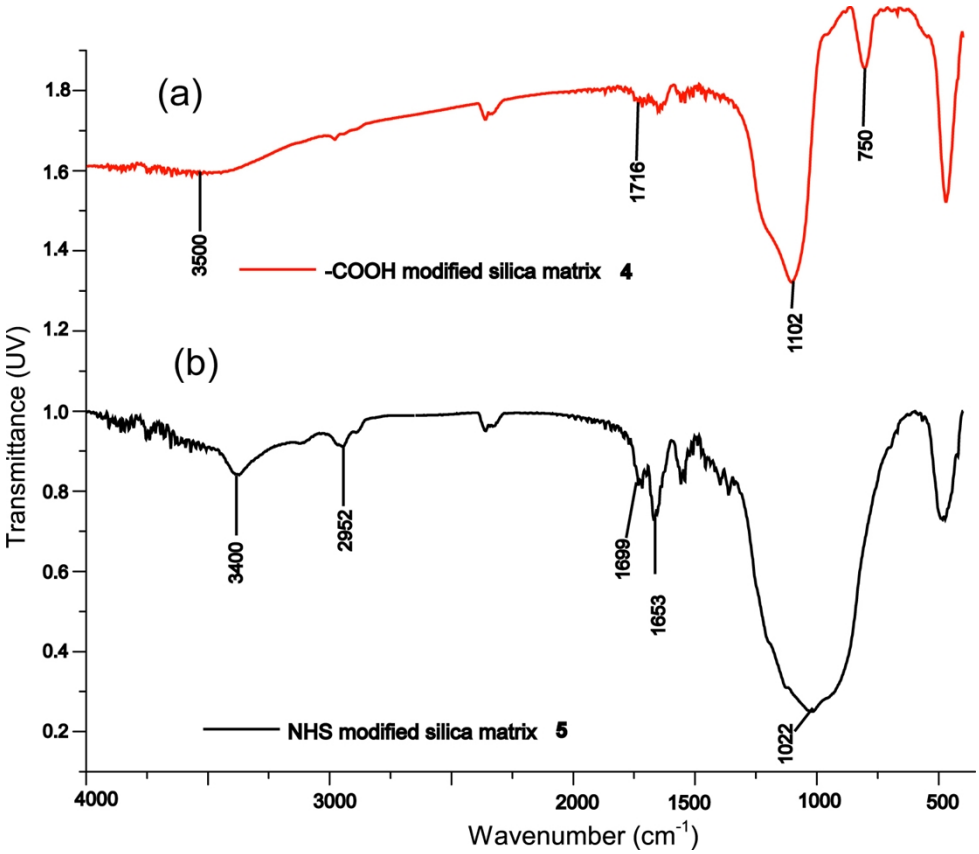

Fig. S2 Fourier Transform Infrared Spectroscopy (FT-IR) of silica particles **4** and silica particles **5**.

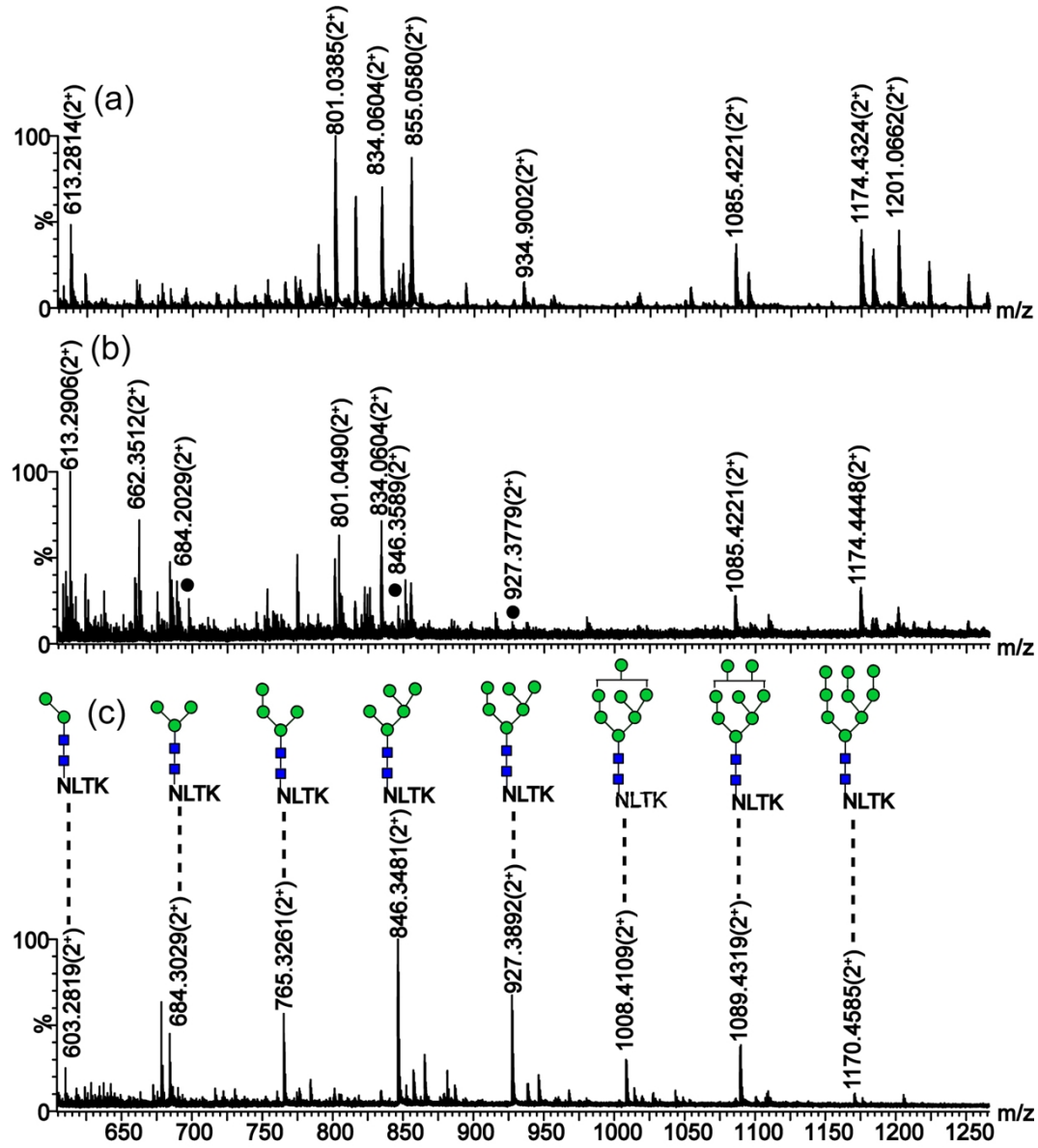

Fig. S3 Mass spectrum of RNase B glycopeptides enriched with Agarose Con A under SPE mode. (a) loading sample with 20 mM HEPES buffer (pH 7.2), desalted; (b) washing sample with 20 mM HEPES buffer (pH 7.2), desalted with C18 and enriched with Click Maltose; (c) eluted sample with 0.2 M methyl α-D-mannopyranoside in 20 mM HEPES buffer (pH 7.2), desalted with C18 and enriched with Click Maltose.

● Mannose; ■ Nacetylglucosamine

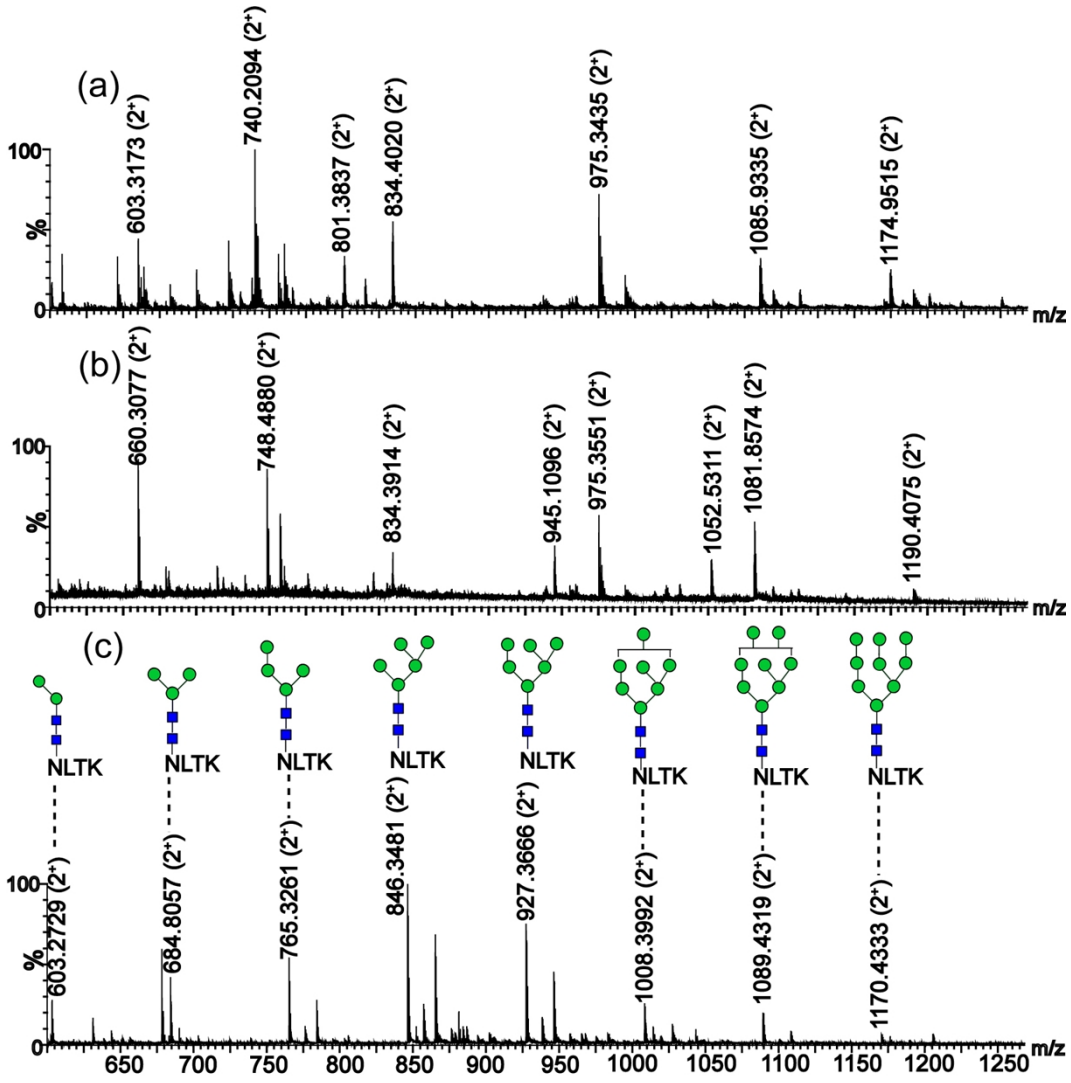

Fig. S4 Mass spectrum of RNase B glycopeptides enriched with SG ConA. (a) loading sample with 10 mM HEPES buffer (pH 7.2), desalted with C18; (b) washing sample with 10 mM HEPES buffer (pH 7.2), desalted with C18 and enriched with Click Maltose; (c) elution of sample with 0.2 M methyl α-D-mannopyranoside in 10 mM HEPES buffer (pH 7.2), desalted with C18 and enriched with Click Maltose.

● Mannose; ■ Nacetylglucosamine

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