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Selective enrichment of glycopeptides / phosphopeptides using porous titania microspheres

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Experimental Section

Materials and Chemicals: α-casein, horseradish peroxidase (HRP, 98%), bovine ribonuclease B (RNase B), human serum immunoglobulin G (IgG) serum albumin from human (HSA), Sepharose CL-6B, ammonium bicarbonate (NH_4HCO_3), lactic acid (LA), dithiothreitol (DTT) and iodoacetic acid (IAA) were obtained from Sigma-Aldrich (St. Louis, MO). Formic acid (FA) was purchased from Acros Organics (Geel, Belgium). Trifluoroacetic acid (TFA, 99.8%) was obtained from TEDIA (Fairfield, USA). Trypsin was from Promega (Madison, WI). Reversed phase C_{18} particles were from Sunchrom (Friedrichsdorf, Germany). GELoader tips were obtained from Eppendorf (Hamburg, Germany). Ammonium hydroxide (NH_4OH) was purchased from Fluka (Buchs, Switzerland). Acetonitril (ACN) was from Merck (Darmstadt, Germany). All these reagents were used as received without further purification. Water used in MS was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

Preparation of titania microspheres: $Ti(OBu)_4$ (8.0 g), acetonyl acetone (1.5 mL) and dodecylamine (2.0 g) were stirred vigorously in anhydrous ethanol (200 mL) at 30 ℃. Certain amount of water was slowly added to the mixture and stirred for another 4 minutes until the solution became cloudy. 50 mL anhydrous ethanol was added to the turbid solution. The mixture was aged about 2 h at 35 ℃. Then the sediment was filtered and washed with anhydrous ethanol and dried at room temperature for 12 h. The obtained xerogel (2.0 g), urea (0.1 g), anhydrous ethanol (21 mL) and deionized water (14 mL) were sealed in a PTFE-lining stainless-steel autoclave and heated at 135 ℃ for 24 h. The particles were separated and washed with acetone and methanol for several times. Then the micropheres were calcinated at 200 ℃ (at a heating rate of 5℃ min^{-1}) keeping for 3 h and at 400 ℃ for 6 h to remove the residuary organic compound.

Digestion with trypsin: 1 mg of proteins except for IgG (0.5mg) and HSA (0.5mg) were dissolved in 100 μL of denaturing buffer containing 8M urea and 50 mM ammonium bicarbonate and incubated for 3 h. The obtained protein solution was mixed with 2 μL of 500 mM DTT. The disulfide bond of protein was reduced by incubation for 2 h at 37 ℃. Then 4 μL of 500 mM IAA was incubated for additional 30 min at room temperature in the dark. After that, the mixture was diluted with 50 mM ammonium bicarbonate by ten-fold and incubated for 16 h at 37 ℃ with trypsin at an enzyme / substance ratio of 1:25 (w/w). Final concentration 0.5% FA was used to

stop the digest. Phosphoprotein α-casein were dissolved in 1 mL of ammonium bicarbonate (50 mM, pH 8.0) and digested in trypsin for 18 h at 37 ℃ with a 1:40 (w/w) enzyme to protein ratio.

Enrichment of glycopeptides: 1 mg of TiO_2 beads were packed in GELoader tips. The tryptic digests were dried, redissolved in the same volumn of ACN/H_2O/FA (50/50/0.1 (v/v)) solution as loading buffer. RNase B tryptic digest (5 μL, 300 pmol) was loaded on TiO_2 column. The column was washed with ACN/H_2O/FA (50/50/0.1 (v/v), 45 μL) three times. Glycopeptides of RNase B were eluted with 10 μL of 0.1%FA two times; the elution solvent was collected and directly infused to MS. IgG tryptic digest (140 pmol) was dissolved in 10 μL of loading buffer and loaded on the TiO_2 tip. The column was successively washed with ACN/H_2O/FA (50/50/1 (v/v), 45 μL) solution three times and 45 μL of 1% FA two times, the bound glycopeptides were eluted with 20 μL of 5%TFA three times. The eluted solution was neutralized with 10% NH_4OH and desalted with C18 microcolumn before MS analysis. Enrichment procedure of Sepharose followed a published protocol. {Ref 1}

Ref 1

[7] L. Yu, X. L. Li, Z. M. Guo, X. L. Zhang and X. M. Liang, *Chem. Eur. J.*, 2009, **15**, 12618.

Simultaneous enrichment of glycopeptides and phosphopeptides from standard protein mixture: The mixture of HRP and α-casein and HSA digest solution (1:1:1(mol/mol), 50pmol) redissloved in 10 μL of ACN/H_2O/FA (50/50/0.1 (v/v)) solution and loaded on the TiO_2 tip. After successive washing with ACN/50mM $NH4HCO_3$ aqueous solution (50/50 (v/v), 45μL) three times and ACN/H_2O/FA (50/50/0.1 (v/v), 45μL) two times, the bound glycopeptides were eluted with 45 μL of 10% ACN with 5% FA three times. The glycopeptides fraction were collected, dried and redissolved in 10μL of ACN/H_2O/FA (50/50/0.1 (v/v)) for MS analysis. The glycopeptides were identified according to the report [6g] and MS/MS (Figure S2). In order to remove glycopeptides and non-phosphopeptides with the greatest degree, 300mg mL^{-1} LA in ACN/H_2O/FA (10/90/5 (v/v)) solution and 5%FA aqueous solution was used after glycopeptides fraction. Finally, 10% NH_4OH was used to elute phosphopeptides. The eluted solution was acidified with TFA solution and desalted with C18 microcolumns before MS analysis.

Nano-ESI-MS analysis: The glycopeptides solution after enrichment was infused into a quadrupole time-of-flight (Q-Tof) mass spectrometer (Waters, Manchester, UK) at a flow rate of 1 μL min^{-1}. Each spectrum was collected over 0.5s scans, and the spectra were accumulated over 1min. The instrument was controlled by MassLynx 4.1 software. Argon gas was used as the collision gas with different voltage values of 20-50V between the collision cell and the coming ions.

Characterization

The morphologies and elemental analysis of particles were studied using scanning electron microscopy (Jeol JSM-6360LV, Jeol Ltd., Japan). The isotherms were analyzed by nonlocal density functional theory (NLDFT) method to evaluate pore sizes and micropore volumes of the samples using the kernel of NLDFT equilibrium capillary condensation isotherms of nitrogen at 77 K on silica (adsorption branch, assuming cylindrical pore geometry). The BET surface areas were calculated from the adsorption branches in the relative pressure range of 0.05-0.20, and the total pore volumes were evaluated at a relative pressure of 0.95.

Tables and Figures

Table S1 Porous properties of titania microspheres

sample	BET surface area / m^2g^{-1}	Pore volume / m^3g^{-1}	Pore diameter / nm
TiO_2	76.8	0.204	10.8

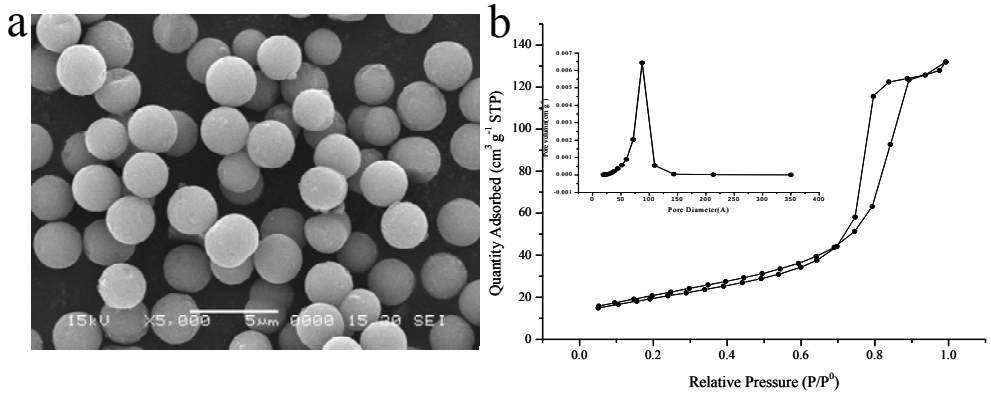

Figure S1. (a) SEM image of homemade TiO_2 microspheres. (b) N_2 adsoption and desorption isotherms and corresponding pore size distributions.

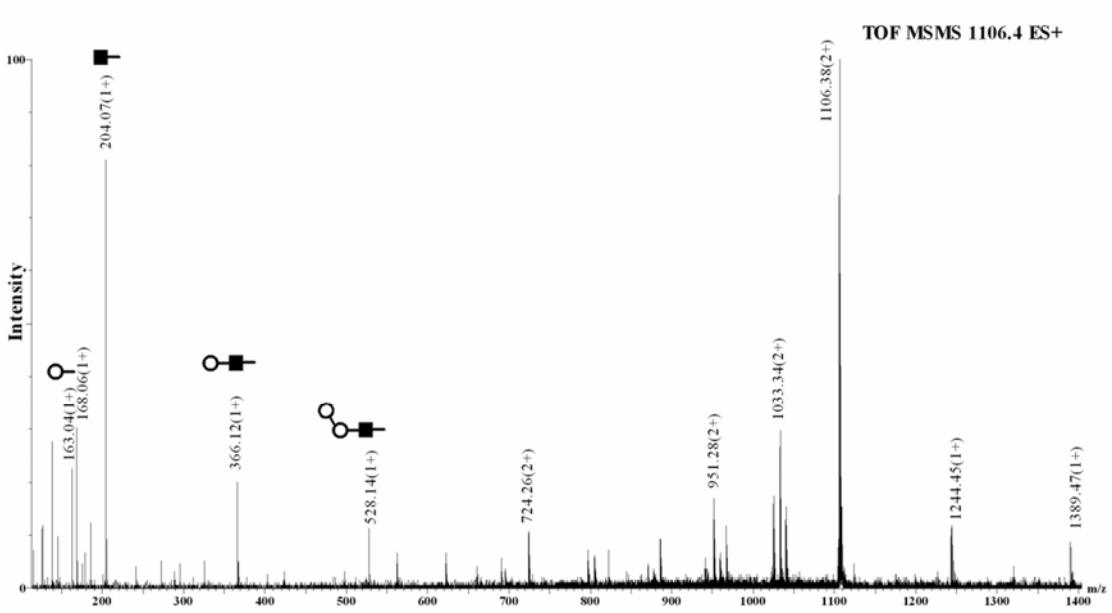

Figure S2-1. The ESI-MS/MS mass spectra of double charged N-glycosylated peptide at m/z 1106.4. Key: ○ mannose; ■ N-acetyl-glucosamine.

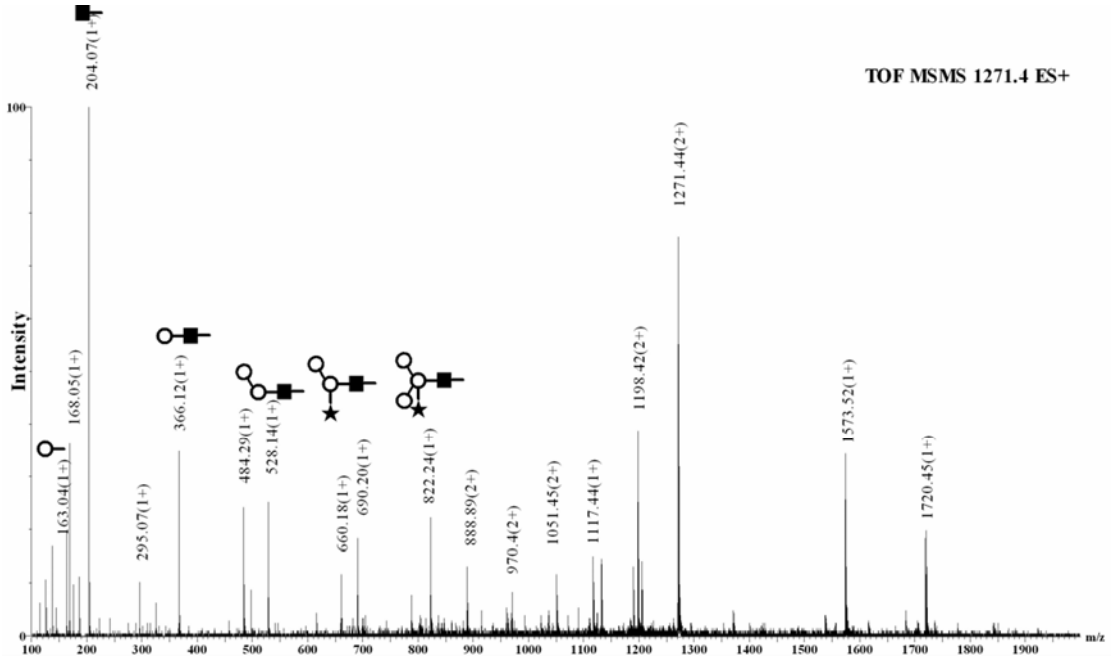

Figure S2-2. The ESI-MS/MS mass spectra of double charged N-glycosylated peptide at m/z 1271.4. Key: ○ mannose; ■ N-acetyl-glucosamine; ★ xylose.

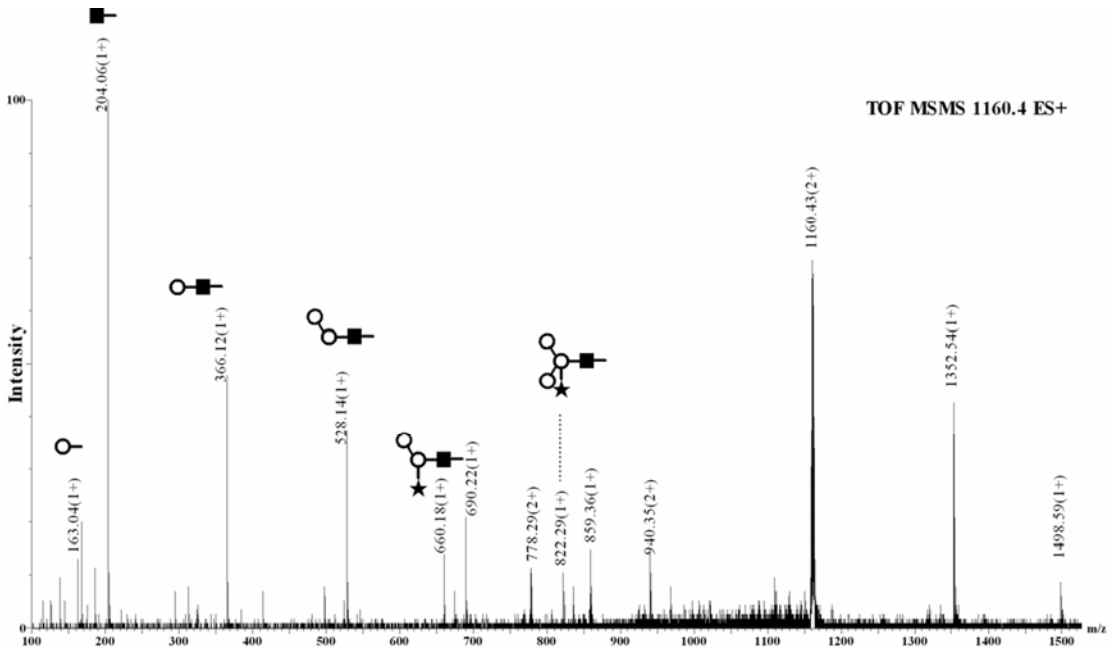

Figure S2-3. The ESI-MS/MS mass spectra of double charged N-glycosylated peptide at m/z 1160.4. Key: ○ mannose; ■ N-acetyl-glucosamine; ★ xylose.