Hydrophilic GO/Fe_3O_4/Au/PEG nanocomposites for highly selective enrichment of glycopeptides

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Experimental

Chemicals and materials

Graphene oxide was supported by Chen Yongsheng group (NanKai University, Tianjin, China). Amine-functionalized magnetic nanoparticles were purchased from Nano-Micro technology (Suzhou, China). Polyethylenimine (PEI, Mn:10000),Thiol-end polyethylene glycol (SH-PEG, Mn: 6000), 2-Morpholino-ethanesulfonic acid (MES), TPCK-treated trypsin, myoglobin (Myo), horseradish peroxidase (HRP), human serum immunoglobulin G (human IgG), dithiothreitol (DTT), iodoacetamide (IAA), N-hydroxysulfosuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), formic acid (FA), urea and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). 2, 5-dihydroxybenzoic acid (DHB) was obtained from Bruker (Daltonios, Germany). Acetonitrile (ACN) was of HPLC grade purchased from Merck (Darmstadt, Germany). Peptide-N-glycosidase (PNGase F) was obtained from New England Biolabs (Ipswich, MA, USA). Multiple affinity removal column (4.6×50 mm, Hu-14) and buffers were obtained from Agilent Technologies (Agilent, California, USA). Deionized water was purified using a Milli-

Q system (Millipore, Molsheim, France). All other reagents were of analytical grade purchased from China.

Characterization

Fourier-transformed infrared spectroscopy (FT-IR) characterization has been performed on Perkin-Elmer Spectrum GX spectrometer (Perkin-Elmer, Waltham, USA). Transmission electron microscopy (TEM) images were obtained by JEOL JEM-2000EX instrument operated at 120 kV (JEOL, Tokyo, Japan). X-ray photoelectron spectroscopy (XPS) measurements were conducted with Thermo ESCALAB250Xi spectrometer with Al Kα radiation as the X-ray source (Thermo, Waltham, USA). The potential of nanoparticles was detected by Malven Nano-ZS90 dynamic light scattering (Malven, Worcester, UK). Thermo Gravimetric Analysis (TGA) was conducted with Netzsch STA449F3 thermal analyzer (Netzsch, Bavaria, Germany) that was fitted to a nitrogen purge gas at 10 ºC/min heating rate. The Nitrogen adsorption and desorption isotherms were measured by QuadrasorbSI (Quadraorb, Wisconsin, USA). The Brunauer-Emmett-Teller (BET) method was used to calculate the specific surface areas with adsorption data in a relative pressure range from 0.053 to 0.248.

Preparation of GO/Fe_3O_4/Au/PEG nanocomposites

Magnetic graphene oxide (GO/Fe_3O_4) nanocomposites were synthesized via covalent reaction under ambient conditions. Firstly, GO (10 mg) in 40 mL of MES buffer (0.1 M, pH 5.6) was ultrasonicated for 3 h, and then 95.5 mg of EDC and 57.5 mg of NHS were added into the suspension of exfoliated GO. The above mixture was then ultrasonicated for 2 h to activate carboxyl groups of GO. Finally, 20 mg of amine-functionalized magnetic nanoparticles were added into the suspension, and ultrasonicated for another 1 h. GO/Fe_3O_4 was separated from reaction mixtures by using external magnetic force and washed with deionized water for several times. The prepared GO/Fe_3O_4 nanocomposites were dried under vacuum at 60 ºC.

GO/Fe_3O_4 nanocomposites were uses as support for immobilizing gold

nanoparticles (Au NPs). In a typical process, 30 mg PEI was added into 1 mL GO/Fe_3O_4 suspension (1.5 mg/mL) under vigorous stirring for 1 h to obtain homogenous suspension. The product was separated from reaction mixtures by using external magnetic force and redissolved in 1 mL deionized water. Then, 4 μL (100 mg/mL) $HAuCl_4.3H_2O$ solutions were added to the above suspension. The resulting suspension was heated at 70 ºC for 1 h to generate AuNPs. After magnetic separation, the resulted GO/Fe_3O_4/Aunanocomposites were vacuum-dried at 60 ºC for further use. In the next step, SH-PEG was applied to improve the hydrophilicity of GO/Fe_3O_4/Au nanocomposites. Two mg GO/Fe_3O_4/Au were mixed with 20 mg thiol-end PEG in 1 mL deionized water at room temperatureunder stirring for 24 h. The prepared GO/Fe_3O_4/Au/PEG nanocomposites were separated and washed with deionized water three times to remove excess thiol-end PEG. The produce was vacuum-dried at 60 ºC for glycopeptides enrichment.

Human serum high-abundant proteins removal

Agilent multiple affinity removal column was used to remove 14 high-abundant proteins from 20 μL original human plasma. The obtained human plasma were desalted by C8 trap column and dissolved in 50 mM NH_4HCO_3 (pH 8.0) containing 8 M urea. The concentration of the proteins was determined as 1 mg/mL by BCA reagents.

Sample preparation

HRP (1 mg/mL) was dissolved in 1 mL of NH_4HCO_3 (pH 8.0, 50 mM) and denatured at 90 °C for 10 min. Then, TPCK-treated trypsin was added to a final volume of 1:40 w/w of glycoprotein. The tryptic digestion proceeded at 37 ºC for 12 h. For Myo, the concentration was decreased to 0.5 mg/mL. Human IgG and human plasma proteins were dissolved in 50 mM NH_4HCO_3 (pH 8.0) containing 8 M urea, and then reduced in 10 mM DTT for 1 h at 56 °C. When cooled to room temperature, cysteines were alkylated in the dark in 20 mM IAA for 30 min at 37 °C. After being diluted ten-fold with 50 mM NH_4HCO_3 (pH 8.0), the solution was subsequently

treated with trypsin at 37 °C (enzyme/protein ratio of 1:40, w/w) for 12 h. The tryptic digestions were desalted on an SPE-C18 column with 2% and 98% ACN (v/v), containing 0.1% TFA (v/v), as the loading and eluting buffer, respectively. The eluant was lyophilized in a SpeedVac (Thermo Fisher, San Jose, CA, USA), and stocked at -20ºC for further use.

Hydrophilic enrichment of glycopeptides

The process for enrichment of glycopeptides was illustrated in Scheme S1. For tryptic HRP, 100 μg GO/Fe_3O_4/Au/PEG nanocomposites were added to 0.1 μg tryptic digests dissolved in 50 μL ACN/H_2O/FA solution (80: 20: 0.1, v/v/v, containing 10 mM NH_4HCO_3). The capture procedure was carried out under gentle agitation at room temperature for 1 min. After adsorption, the supernatant was discarding by magnetic separation. The nanocomposites were washed three times with loading buffer to remove the non-glycopeptides (3×20 μL). Glycopeptides were eluted with 2×20 μL ACN/H_2O/FA (50: 50: 0.1, v/v/v, containing 10 mM NH_4HCO_3). For human IgG (contain sialic acid), 75% ACN/20 mM NH_4HCO_3 and 25 mM NH_4HCO_3 were selected as loading and eluting buffers, respectively. The eluants were lyophilized in a SpeedVac (Thermo Fisher, San Jose, CA, USA), and redissolved in 1 μL DHB solution (20 mg/mL, 0.1% TFA in 60% ACN). For human serum, 400 μg GO/Fe_3O_4/Au/PEG nanocomposites were added to 20 μg tryptic digests dissolved in 100 μL ACN/H_2O/FA solution(75: 25: 0.1, v/v/v, containing 10 mM NH_4HCO_3). The capture procedure was carried out under gentle agitation at room temperature for 10 min. After adsorption, the supernatant was discarding by magnetic separation. The nanocomposites were washed three times with loading buffer to remove the non-glycopeptides (3×100 μL). Glycopeptides were eluted with 2×25 μL ACN/H_2O/FA (50: 50: 0.1, v/v/v, containing 10 mM NH_4HCO_3). For click maltose, the same procedure was applied to enrich glycopeptides from tryptic human serum.

Deglycosylation of glycopeptides by PNGase F

Each glycopeptides fraction collect from the enrichment was redissolved in 50

μL 25 mM NH_4HCO_4 solution, 1000 U of PNGase F was added to the solution, and incubated overnight at 37 °C for N-glycan release. Then deglycosylation peptides were directly spotted on the MALDI target plate or analysis by *nano*-LC-MS/MS.

Recovery estimation of glycopeptides enrichment

Two of the same amounts of human IgG (10 μg) digest were firstly labeled with light and heavy isotopes by using a stable isotope dimethyl labeling approachaccording to previously reported procedure.The heavy-tagged human IgG digest was enriched with GO/Fe_3O_4/Au/PEG according to above-mentioned procedure and the resulting eluted fraction was spiked into light-tagged human IgG digest. The combined mixture was re-enriched with GO/Fe_3O_4/Au/PEG, and the eluted fraction was deglycosylatedand analyzedby MALDI-TOF MS. The recovery was calculated by the peak intensity ration of heavy isotope-labeled deglycopeptidesto the light isotope-labeled glycopeptides.

MS analysis and date research

All MALDI spectra were taken from a Bruker Ultraflex III MALDI-TOF/TOF MS instrument (Bruker, Daltonios, Germany). A total of 1 μL of elution was dropped onto a MALDI plate, to which 1 μL of DHB solution was added. The laser intensity was kept constant for all samples. External calibration of MALDI-TOF/TOF MS spectra was performed with ten commercial peptides. The obtained human plasma glycopeptides were injected for *nano*-LC-MS/MS analysis. A packed C_{18} column (75 μm i.d.× 15 cm) was used for peptide separation, with the flow rate of 200 nL/min. Two percent (v/v) ACN with 0.1% (v/v) FA (buffer A) and 98% (v/v) ACN with 0.1% (v/v) FA (buffer B) were used to generate a 125 min gradient, set as follows: 0% B for 10 min, to 5% B in 15 min, to 35% B in 105 min, to 80% B in 115 min, and kept at 80% B for 10 min. The LTQ-Orbitrap instrument (Thermo-Fisher, San Jose, CA, USA) was operated at positive ion mode. The spray voltage was 2.3 kV, and the heated capillary temperature was 200°C. Total ion current chromatograms and mass spectra covering the mass range from m/z 350 to 1800 were recorded with Xcalibur software

(version 1.4). MS/MS spectra were acquired by data-dependent acquisition mode with 15 precursor ions selected from one MS scan. Precursor selection was based on parent ions intensity, and the normalized collision energy for MS/MS scanning was 35%.

The acquired MS/MS spectra were searched against the International Protein Index (IPI) human protein database (version 3.71) using MASCOT software (version 2.3.2). The search criteria were set as follows: variable modifications of methionine oxidation (+16Da), deamidation (N)and fixed modification of cysteine residues (+57 Da), at most two missed tryptic cleavage sites,10 ppm error tolerance in MS and 0.5 Da error tolerance in MS/MS. The search results were filtered by pBuild to control the peptide FDR≤1%. FDRs were calculated by using the following equation: FDR= n(rev)/n(forw), where n(forw) and n(rev) are the number of peptides identified in proteins with forward (normal) and reversed sequence, respectively. Since N-glycosylation occurs at a consensus N-X-S/T (X≠P), the remaining peptide sequences were additionally filtered to remove non-motif containing peptides.

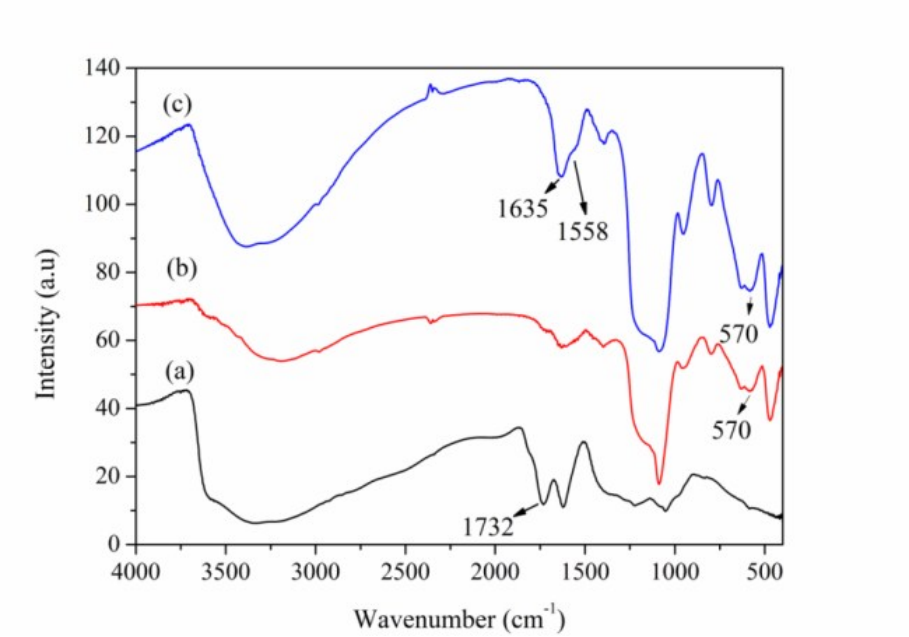


Fig. S1 FT-IR spectra of (a) GO nanosheets, (b) magnetic nanoparticles and (c) GO/Fe_3O_4 nanocomposites.

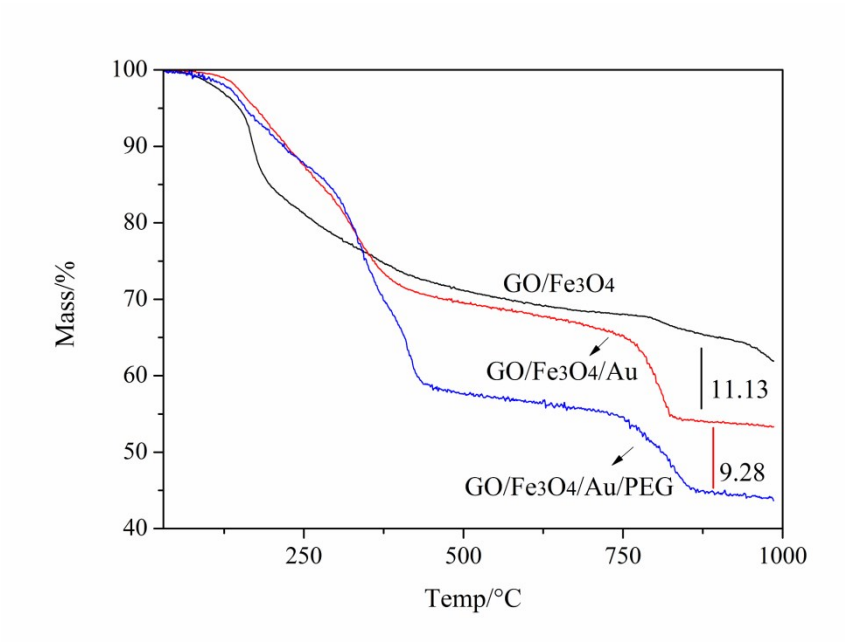


Fig. S2 TGA curves of GO/Fe_3O_4, GO/Fe_3O_4/Au and GO/Fe_3O_4/Au/PEG nanocomposites.

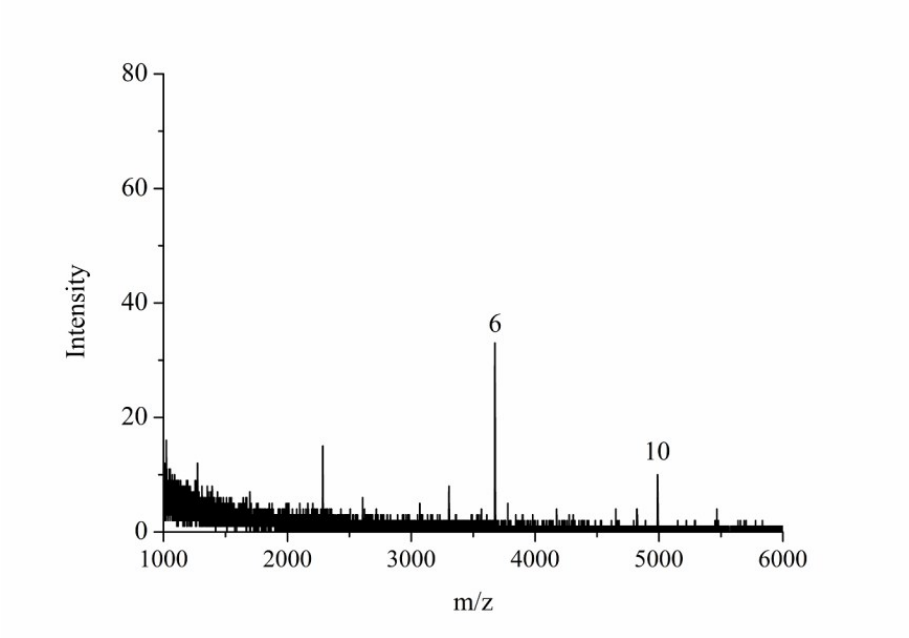
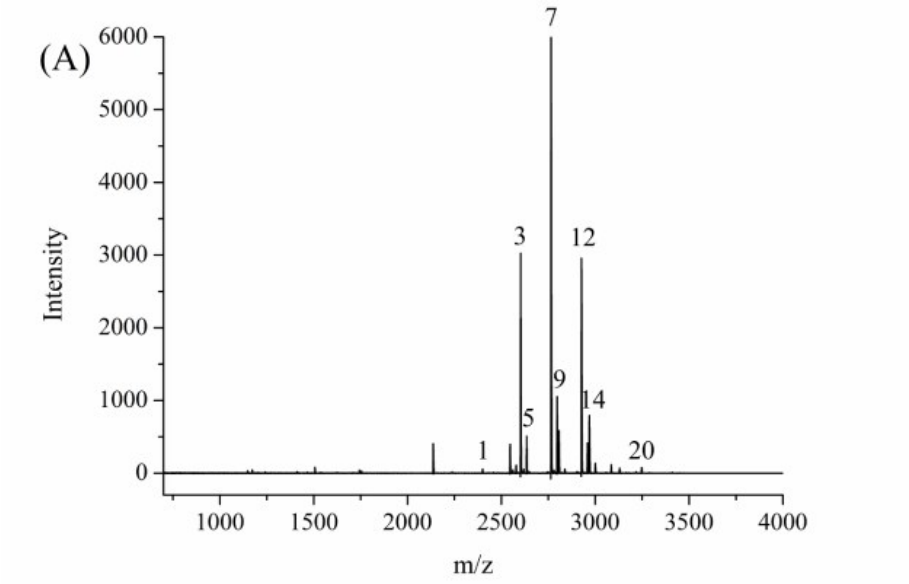


Fig. S3 MALDI-TOF MS spectra of 25 fmol tryptic HRP after enrichment by GO/Fe_3O_4/Au/PEG nanocomposites.



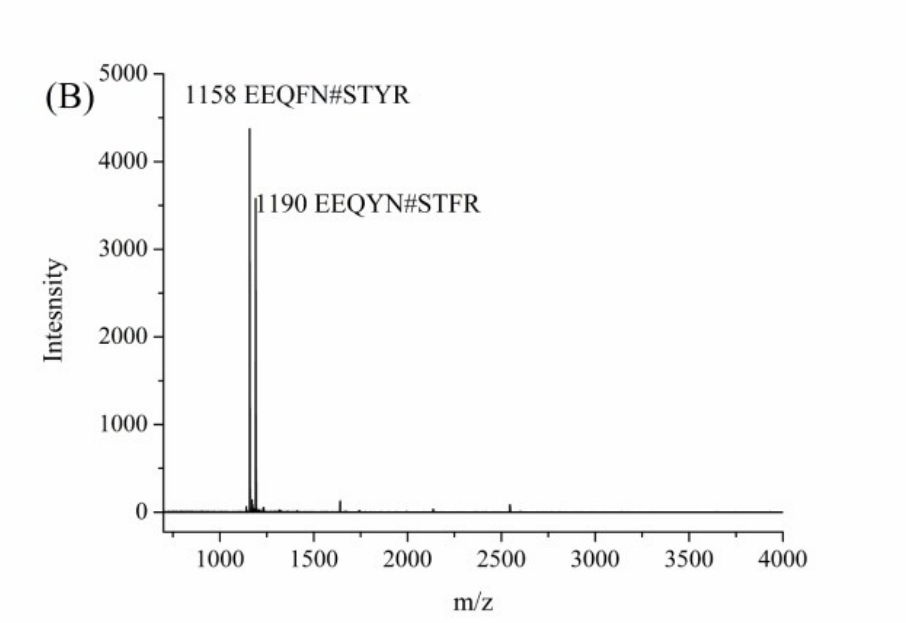
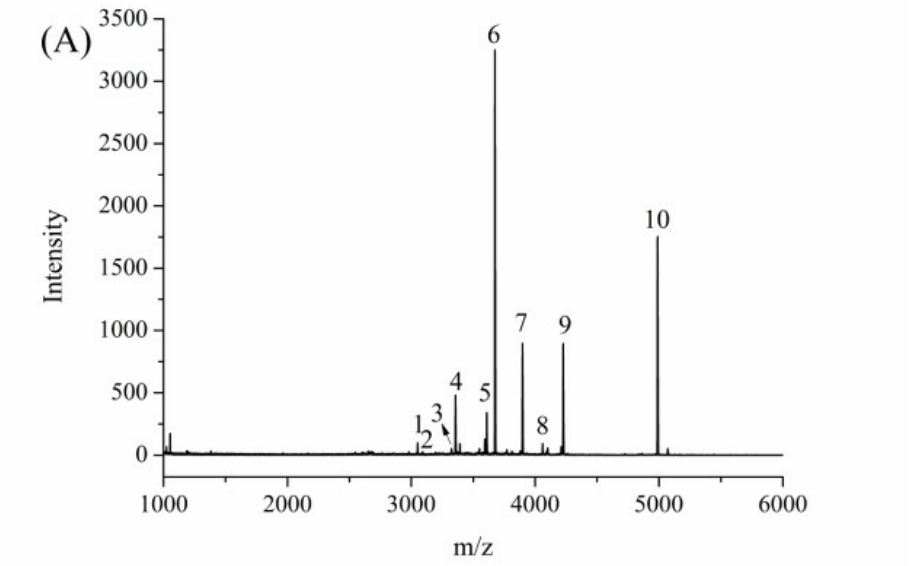


Fig. S4 MALDI-TOF MS spectra of 1 pmol tryptic digest of human IgG after enrichment with (a) GO/Fe_3O_4/Au/PEG composites and (b) the deglycosylated peptides by PNGase F.



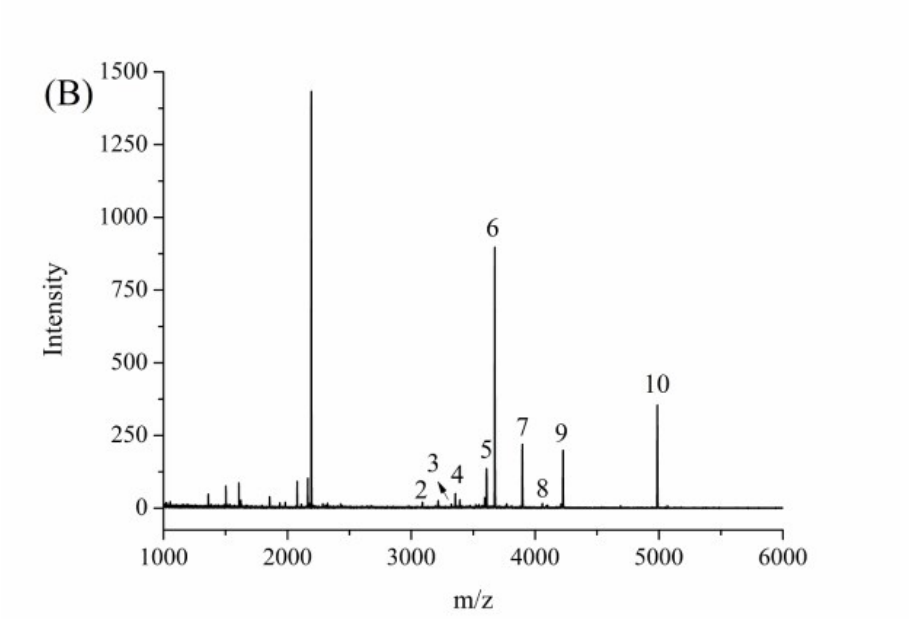


Fig. S5 MALDI-TOF MS spectra of the enriched glycopeptides from the tryptic digest mixture of HRP and Myo with the mass ratio of (A) 1:10 and (B) 1:100.

Table S1. Molecular masses and proposed oligosaccharide composition of the glycopeptides from HRP after enrichment. N# denotes the N-linked glycosylation site.

Number	m/z	Glycan composite	Amino acid sequence
1	3050	[Hex]2[HexNAc][Xyl]1	SFAN#STQTFFNAFVEAMDR
2	3089	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	GLCPLNGN#LSALVDFDLR
3	3322	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	QLTPTFYDNSCPN#VSNIVR
4	3354	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	SFAN#STQTFFNAFVEAMDR
5	3607	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	NQCRGLCPLNGN#LSALVDFDLR
6	3673	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	GLIQSDQELFSSPN#ATDTIPLVR
7	3895	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	LHFHDCFVNGCDASILLDN#TTSFR
8	4057	[Hex]3[HexNAc]2[Xyl]1	QLTPTFYDNSC(AAVESACPR)PN#VSNIVR-H_2O
9	4224	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	QLTPTFYDNSC(AAVESACPR)PN#VSNIVR
10	4985	[Hex]3[HexNAc]2[Fuc]1[Xyl]1 [Hex]3[HexNAc]2[Fuc]1[Xyl]1	LYN#FSNTGLPDPTLN#TTYLQTLR

HexNAc=N-acetylglucosamine, Fuc=fuctose, Hex=mannose,Xyl=xylose.

Table S2. Molecular masses and proposed oligosaccharide composition of the glycopeptides from human IgG after enrichment. N# denotes the N-linked glycosylation site.

Number	m/z	Glycan composite	Amino acid sequence
1	2400	[Hex]3[HexNAc]3[Fuc]1	EEQFN#STYR
2	2561	[Hex]4[HexNAc]3[Fuc]1	EEQFN#STYR
3	2603	[Hex]3[HexNAc]4[Fuc]1	EEQFN#STFR
4	2618	[Hex]4[HexNAc]4	EEQFN#STFR
5	2635	[Hex]3[HexNAc]4[Fuc]1	EEQYN#STYR
6	2650	[Hex]4[HexNAc]4	EEQYN#STYR
7	2764	[Hex]4[HexNAc]4[Fuc]1	EEQFN#STFR
8	2780	[Hex]5[HexNAc]4	EEQFN#STFR
9	2797	[Hex]4[HexNAc]4[Fuc]1	EEQYN#STYR
10	2806	[Hex]3[HexNAc]5[Fuc]1	EEQFN#STYR
11	2838	[Hex]3[HexNAc]5[Fuc]1	EEQYN#STYR
12	2926	[Hex]5[HexNAc]4[Fuc]1	EEQFN#STFR
13	2958	[Hex]5[HexNAc]4[Fuc]1	EEQYN#STYR
14	2968	[Hex]4[Hex7NAc]5[Fuc]1	EEQFN#STFR
15	3000	[Hex]4[HexNAc]5[Fuc]1	EEQYN#STYR
16	3087	[Hex]4[HexNAc]4[Fuc]1[NeuAc]1	EEQYN#STFR
17	3129	[Hex]5[HexNAc]5[Fuc]1	EEQFN#STFR
18	3161	[Hex]5[HexNAc]5[Fuc]1	EEQYN#STYR
19	3218	[Hex]5[HexNAc]4[Fuc]1[NeuAc]1	EEQFN#STFR
20	3250	[Hex]5[HexNAc]4[Fuc]1[NeuAc]1	EEQYN#STYR

HexNAc=N-acetylglucosamine,Fuc=fuctose, Hex=mannose,Xyl=xylose, NeuAc=Sialic.