Synthesis of branched PEG brushes hybrid hydrophilic magnetic nanoparticles for the selective enrichment of N-linked glycopeptides

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Experiment details

Chemicals and reagents

Tetraethoxysilane (TEOS), 3-aminopropyltrimethoxysilane (APTMOS), human serum immunoglobulin G (human IgG), horseradish peroxidae (HRP), human alpha-acid glycoprotein (human AGP)**,** 2,2'-bipyridyl, copper (I) chloride, 2-bromoisobutyryl bromide, poly (ethylene glycol) methacrylate (PEG, Mn:~ 360), trypsin, elastase, chylmotrypsin, Gluc and 2, 5-dihydroxybenzoic acid (2, 5-DHB) were purchased from sigma-Aldrich (St, Louis, MO, USA). Peptide-N-glycosidase (PNGase F) was obtained from New England Biolabs (Ipswich, MA, USA). Centrifugal filter with MWCO of 3 KDa was purchased from Millipore (Bedford, MA). Urea, tris, dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from BioRad (Hercules, CA, USA). Human plasma was obtained from a healthy donor. Acetonitrile (ACN), formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Iron (Ш) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ammonium solution (25 wt %), sodium acetate anhydrous, triethylamine, isopropanol, dichloromethane and methanol were obtained from Tianjin Kermel plant of chemical reagent (Tianjin, China). Pure water was prepared with a Milli-Q system (Millpore, Bedford, MA, USA). All other chemical and reagents were of analytical grade and were obtained from Shanghai Chemical Reagnet.

Synthesis of hybrid Fe_3O_4@SiO_2@PEG-Maltose MNPs

Synthesis route of Fe_3O_4@SiO_2@PEG-Maltose MNPs was shown in **Scheme 1A**.

The Fe_3O_4 MNPs were synthesized according to a literature procedure. The Fe_3O_4 MNPs (350 mg) were washed twice with ethanol and re-dispersed in ethanol (160 mL) with 0.5 h sonication. After the addition of ammonia (25%, 1.0 mL), water (20.0 mL), and TEOS (1.0 mL), the mixed solution was stirred for 6 h at room temperature. With the using of a magnet, the Fe_3O_4@SiO_2 core-shell MNPs were rinsed with ethanol (50 mL) for three times and resuspended in isopropanol (60 mL), in which APTMOS (1.0 mL) was dropwise added, and the reaction solution was mechanically stirred for 24 h. Then, the amine-modified Fe_3O_4@SiO_2 MNPs were isolated, washed with 50 mL isopropanol and 50 mL dichloromethane for three times, respectively, and redispersed

in 50 ml anhydrous dichloromethane. After the addition of distilled triethylamine (0.711 mL, 5.0 mmol), the whole solution was put in an ice bath under argon atmosphere and cooled to 0 °C. 2-bromoisobutyryl bromide (0.528 mL, 4.2 mmol) was added to the above solution, leaving mechanically stirred for 2.0 h at 0 °C and then at room temperature for 16 h. The synthesized MNPs (denoted as Fe_3O_4@SiO_2-Br) were isolated and washed with dichloromethane, ethanol and water for three times, respectively, finally dried in the vacuum oven overnight at 50 °C.

ATRP was carried out following a reported method. Fe_3O_4@SiO_2-Br (150 mg), poly (ethylene glycol) methacrylate (PEG) (3.26 mL), methanol (5.39 mL) and water (1.34 mL) were added to a Schlenk tube equipped with a mechanical stir bar. The mixed solution was submerged into an ice-salt bath, followed three times of pump-refill cycles. At the third cycle, the flask was filled with argon. CuCl (99.5 mg, 1 mmol), $CuBr_2$ (11.1 mg, 0.05 mmol) and 2,2'-dipyridyl (312 mg, 2 mmol) were quickly added to the Schlenk tube. After two additional pump-refill cycles, the solution were stirred for 12 h at 25 °C. The obtained materials (designed as Fe_3O_4@SiO_2@PEG) were thoroughly washed with water, methanol and acetonitrile.

In a dried flask under nitrogen, the obtained Fe_3O_4@SiO_2@PEG MNPs (50 mg) were dispersed in 30 mL dry tetrahydrofuran with sonication for 30 min; triethylamine (1.4 mL) and methylsulfonylchloride (324 μL, 4.2 mmol) were added and stirred over night. The chlorine-modified MNPs were obtained after washed three times with tetrahydrofuran and N,N-dimethylformamide, respectively. Subsequently, stirring 50 mg chlorine-modified MNPs in 30 mL N,N-dimethylformamide solution containing sodium azide (203 mg, 3.12 mmol) and ammonium chloride (167 mg, 3.12 mmol) for 26 h at 50 °C. The obtained materials (designed as Fe_3O_4@SiO_2@PEG-N_3) were isolated, thoroughly washed with N,N-dimethylformamide, water, and methanol, and dried in the vacuum oven overnight at 50 °C.

1-Propargyl-O-maltose was synthesized as following a reported method. In a dried flask under nitrogen atmosphere, acetyl maltose (10.0 g) was dissolved in 150 mL dichloromethane; propargylalcohol (1.0 mL) was added. The result solution was stirred and cooled to 0 °C, boron trifluoride/ether (3.0 mL) was added dropwise. The reaction was stirred for 1 h and at room temperature for 4 h. potassium carbonate (5.0 g) was added and the solution continuously stirred for 30 min. The unreacted solid was filtered out and the filtrate was concentrated, 1-Propargyl-acetyl maltose (7.55 g, 76% yield) was obtained with *m/z* at 697.2 by MALDI-TOF MS. The obtained solid (1.0 g) was dissolved in 50 mL dry methanol solution, and a freshly prepared sodium

methoxide in methanol (12 mL, 1 mol/L) was slowly added dropwised. Monitoring of the reaction by MALDI-TOF MS indicated complete disappearance of 1-Propargyl-acetyl maltose. The reaction was quenched by neutralization with Amberlite IR-120 resin (H^+ form), the solid was filtered out and concentrated to get oily solid. The crude product was directly used for conjugation to MNPs without further purifications. 1-Propargyl-O-maltose was confirmed with by MALDI-TOF MS (m/z at 402.8 [M^+]).

In a dried flask under nitrogen, Fe_3O_4@SiO_2@PEG-N_3 MNPs (100 mg) were suspended in 4 mL methanol/ water (v/v=1:1) and sonicated for 20 min, 30 μL of freshly prepared solution containing sodium assorbate and copper sulfate (mmol/mmol=100: 200), and 1-propargyl-O-maltose (10 mg) were added. The mixture was shaked powerful over night. The obtained MNPs (designed as Fe_3O_4@SiO_2@PEG-Maltose) were isolated, washed six times with ethanol and pure water, and dried at 40 °C for 24 h.

The synthesis route of Fe_3O_4@SiO_2-Maltose MNPs was shown in **Scheme 1B**. The experiment procedure is similar to **Scheme 1A**.

Characterization

Scanning electron microscope (SEM) image was carried out by JSM-6360 LV scanning electron microscope (Jeol, Mitaka, Japan). Transmission electron microscopy (TEM) image was obtained by JEOL JEM-2000 EX transmission electron microscope (JEOL, Tokyo, Japan). Fourier-transformed infrared spectroscopy (FTIR) characterization has been performed on Thermo Nicolet 380 spectrometer using KBr pellets (Nicolet, Wisconsin, USA). Elemental analyses were performed on Vario EL Ⅲ (Elementar, Hanau, Germany). The saturation magnetization curve was carried out at room temperature on the Physical Property Measurement System 9T (Quantum Design, San Diego, USA).

Preparation of standard protein digests

Standard glycoprotein (human IgG, HRP, AGP, 1 mg, respectively) was dissolved in denaturing buffer containing 8 M urea and 100 mM Tris-HCl buffer (pH 8.2), 10 mM dithiothreitol, and heated at 60 °C for 60 min. The sample was aklylated by the addition of iodoacetamide to a concentration of 20 mM and incubated at room temperature in the dark for 40 min. After being diluted ten-fold with 100 mM Tris-HCl buffer (pH 8.2), the solution was subsequently treated with trypsin at 37°C (enzyme/protein ratio of 1:40, w/w) for 18 h. Digestion was stopped with FA to a

final concentration of 0.5 %, and all of the obtained protein tryptic digests were stored in -20 °C. Other three enzymes for human AGP digest procedure are similar to tryptic digest.

SDA-PAGE and tryptic in-gel digestion

The human plasma sample from healthy donor was thawed at 4 °C and ultrafiltrated through a membrane with MWCO of 3 kDa at 14000 g for 30 min. The collected proteins on the membrane were washed with 500 μL 10 mM ammonium bicarbonate (pH 7.5) for three times and dissolved in 500 μL 10 mM ammonium bicarbonate (pH 7.5). Then, protein from human plasma were separated by SDS-PAGE (12 %) and stained with coomassie brilliant blue. Protein gel pieces were cut into five parts : Ⅰ(0-24 KDa), Ⅱ(24- 28 KDa), Ⅲ(28- 52 KDa), Ⅳ(52- 95 KDa), and Ⅴ(95- KDa) and placed in five microcentrifuge tubes, washed with pure water, 50 mM NH_4HCO_3/ACN (1:1, v/v), and ACN before stripe lucency, respectively. 100 μL ACN was added and incubated for 10 min followed by discard the supernatants, then dried under reduced pressure for 10 min. 1.0 mL of 10 mM DTT (in 50 mM NH_4HCO_3, pH 8.0) was added, incubated for 60 min at 56 °C. The supernatant was removed and 1 mL of freshly prepared 55 mM iodoacetamide in 50 mM NH_4HCO_3 (pH 8.0) was added and incubated in the dark for 45 min. The supernatant was discarded and 1.5 mL ACN was added. The supernatant was discarded and dried under reduced pressure for 10 min. The gel pieces were rehydrated in 25 mM NH_4HCO_3 (pH 8.0) (just enough to cover the gel-pieces) containing 10 ng/μL trypsin and centrifugation for 30 min at 4 °C. The supernatant was removed and 20 μL 25 mM NH_4HCO_3 (pH 8.0) was added. The tryptic digestion was carried out at 37 °C for 18 h and stopped by added 1% FA.

After the tryptic digestion was removed and transferred to a new microcentrifuge tube. ACN/H_2O/TFA (60:39.9:0.1,v/v/v) was added to cover the gel-pieces and incubated for 15 min, then the same volume of ACN was added and incubated for 15 min. After centrifugation, the supernatant was collected and repeated two times. The gel pieces were rehydrated in 25 mM NH_4HCO_3 (pH 8.0) and incubated for 15 min, then the same volume of ACN was added and incubated for 15 min. After centrifugation, the supernatant was collected and repeated two times. The collected supernatants were then lyophilized in speed-vac and stored at -20 °C.

Enrichment of N-linked glycopeptides with hybrid Fe_3O_4@SiO_2@PEG-Maltose MNPs

As shown in **Scheme S1**, First, 5 μL MNPs (4.0 mg/mL) was first washed and dispersed in ACN/H_2O/TFA (88: 11.9: 0.1, v/v/v), 100 μL). Then glycoprotein digests were dissolved in ACN/H_2O/TFA (88: 11.9: 0.1, v/v/v), 100 μL) and added to the suspension in a microcentrifuge tube. The capture procedure was carried out under gentle agitation at room temperature for 10 min. After reaction, the supernatant was discarding by separation with a magnet. Wash three times with ACN/H_2O/TFA (88: 11.9: 0.1, v/v/v, 60 μL) to remove the non-glycopeptides. Human IgG, and HRP glycopeptides were eluted with 2× 10 μL H_2O/TFA (99.9: 0.1, v/v), ACN/H_2O/TFA (30: 69.9: 0.1, v/v/v), and H_2O/TFA (99.9: 0.1, v/v), respectively. The elution buffer was added to release the glycopeptides at room temperature for 10 min. The collected peptides were analysis by MALDI-TOF MS.

Deglycosylation of N-linked glycopeptides by PNGase F

Each N-linked glycopeptides fraction collect from the enrichment was redissolved in 30 μL 50 mM NH_4HCO_3 (pH 7.5), 10 U of PNGaseF was added to the solution, and incubated overnight at 37 °C for N-glycan release. The reaction was stopped by heating to 100 °C for 10min, and then directly spotted on the MALDI target plate or analysis by nano-LC-MS/MS.

Mass spectrometry

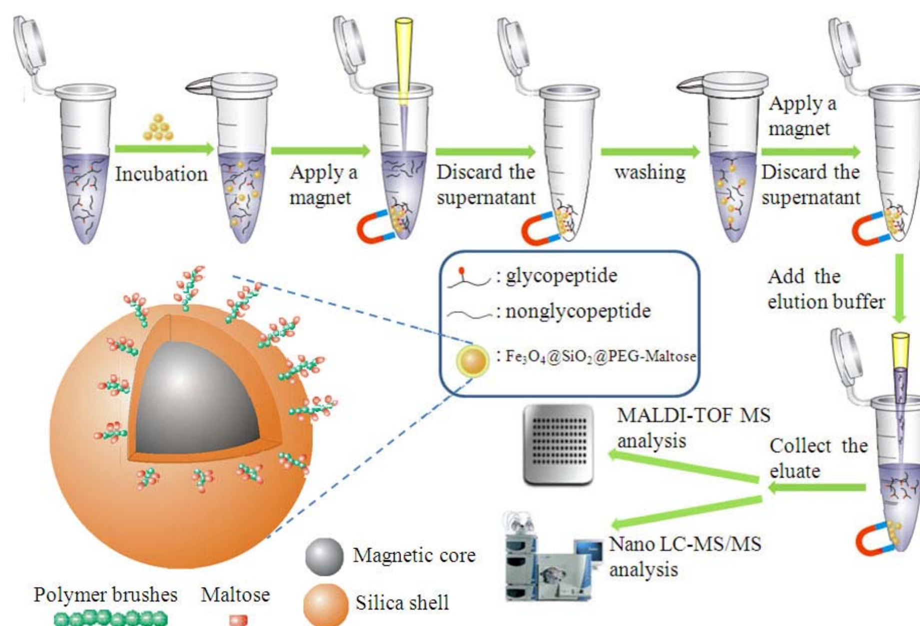
All MALDI-TOF mass spectrometric experiments were carried out on a BRUKER Autoflex time-of-flight mass spectrometer (Bruker Daltoniccs Autoflex, Germany) equipped with a delayed ion-extraction device and a pulsed nitrogen laser (UV, 337 nm). The MALDI-TOF mass spectrometer used a ground-steel sample target with 384 spots. The range of laser energy was adjusted to slightly above the three shold for obtaining the good resolution and signal-to-noise ratio. All measurements were carried out in linear positive-ion mode with delayed ion extraction. The delay time for ion extraction and the extraction voltage were set at 90 ns and 20 kV, respectively. Each TOF-MS spectrum was acquired by the accumulation of 50 laser shots. 2,5-dihydroxy-benzoic acid (DHB) (12.5 mg/mL, in 50% ACN/H_2O solution) was used as the matrix for the analysis of N-linked glycopeptides by MALDI-TOF MS. Sample aliquots (0.5 μL) were placed on MALDI plate and dried at room temperature. After adding DHB matrix (0.5 μL), the sample spots were dried under vacuum prior to TOF-MS analysis.

All nano-LC-MS analyses were performed on a Thermo LTQ-Orbitrap Velos mass spectrometer with a nanospray ion source and a Surveyor HPLC system

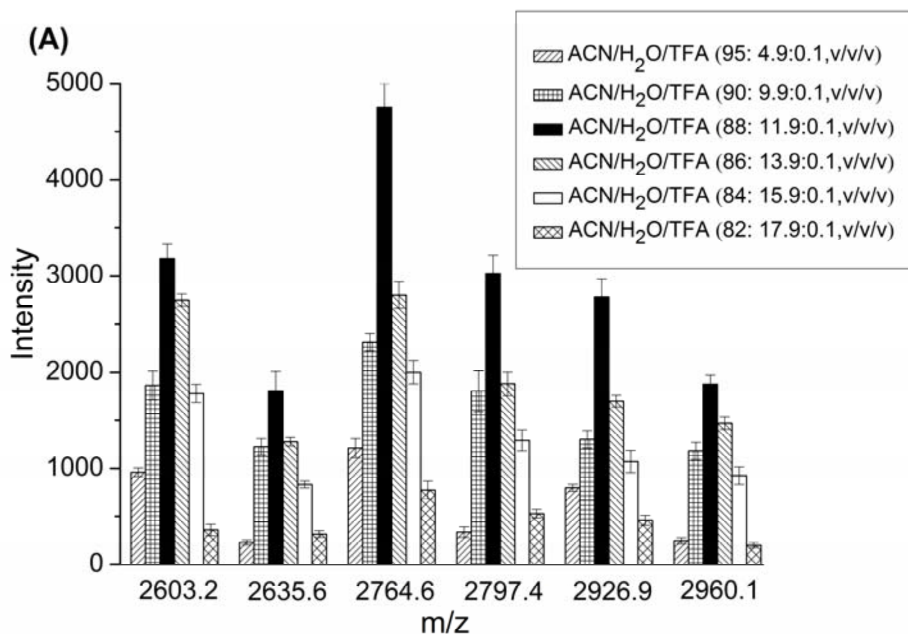
(Thermo, San Jose, CA, USA). Lyophilized enriched glycopeptides were redissolved in H_2O/FA (99.9:0.1, v/v), and injected onto the trap-column with a flow of 100 nL/min. After a valve switch, the trap column was connected to reverse-phase analytical column (120 mm × 75 μm i.d.) packed with C_{18} particles (3 μm, 120 Å). For RPLC separation, H_2O/FA (99.9:0.1, v/v) was used as the mobile phase A, and ACN/FA (99.9:0.1, v/v) was used as the mobile phase B. About 30 μg re-dissolved peptides was loaded onto a C_{18} capillary column as sample loop and separated on the capillary column with a linear gradient where the mobile phase B was programmed from 5 to 35% in 120 min. The flow rate was set at 200 nL/min. The LTQ-Orbitrap mass spectrometer (Thermo, San Jose, CA) was operated in data-dependent MS/MS acquisition mode. Full mass scan was acquired from *m/z* 300 to 2000 (R= 60000 at *m/z* 400). The 20 most intense ions with charge state ≥ 2 and above an intensity threshold of 300 from the full scan were selected to fragmentation in the LTQ. The dynamic exclusion function was set as follows: repeat count 1, repeat duration 30 s, and exclusion duration of 90 s.

Database search and data analysis

The acquired MS/MS spectra were searched against the International Protein Index (IPI) human protein database (version 3.80) using MASCOT software (version 2.3). The search criteria were set as follows: variable modifications of methionine oxidation (+16 Da) and N-terminal acetylation, deamidation (N). and fixed modification of cysteine residues (+57 Da), at most two missed tryptic cleavage sites, 10 ppm error tolerance in MS and 0.8 Da error tolerance in MS/MS. The resulting data files were exported with the filtrations of significance threshold $p < 0.01$ and ion score ⩾25. Since N-glycosylation occurs at a consensus N-X-S/T(X≠P) sequon, the remaining peptide sequences were additionally filtered to remove non-motif containing peptides.



Scheme S1 The application of hybrid $Fe_3O_4@SiO_2@PEG$-Maltose MNPs for the enrichment of glycopeptides with the help of an external magnet field.



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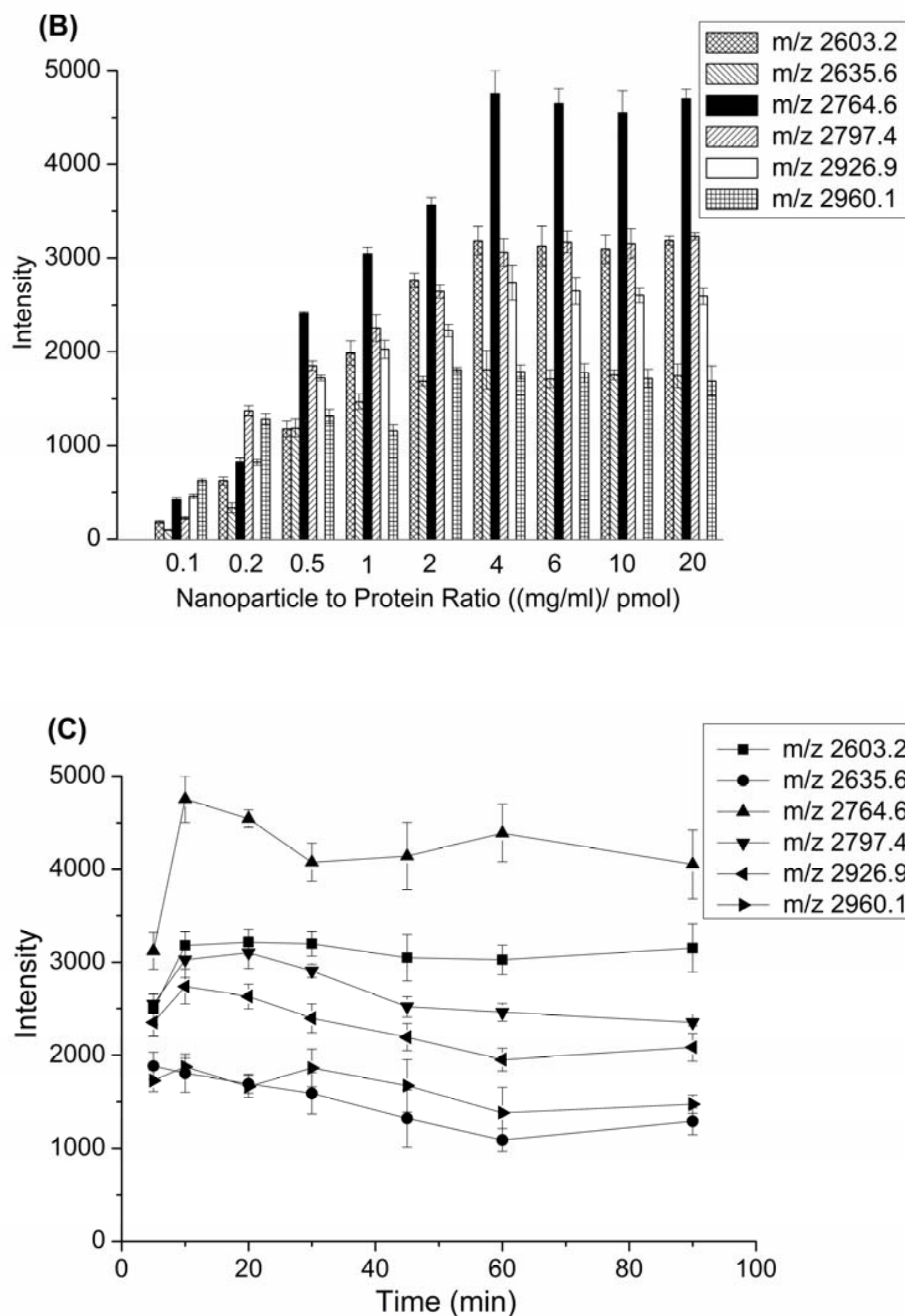

Fig. S1 The effect of A) acetonitrile concentration, B) the ratio of nanoparticle-to-protein ([mg/mL]/pmol) and C) incubation time influencing intensity on peaks of six chosen glycopeptides captured by Fe_3O_4@SiO_2@PEG-Maltose MNPs from tryptic digest of human IgG (0.5 μL, 5 pmol).

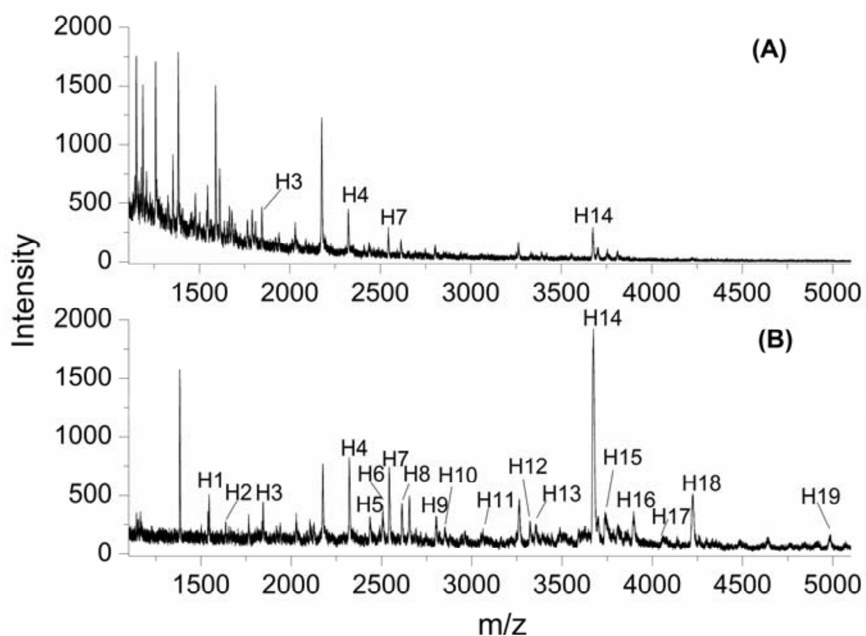

Fig. S2 MALDI-TOF MS spectra of A) direct analysis of 3 pmol tryptic digest of HRP (0.5μL) and (B) after enrichment with Fe_3O_4@SiO_2@PEG-Maltose MNPs.

Alpha-1-acid glycoprotein:

MALSWVLTVLSLLPLLEAQIPL*CANLVPVPITN#ATLDQIT*GKWFYI*ASAFRNEEY N#KSVQEIQATFFYFTPN#KTEDTIFLREYQTRQDQCIYN#TTYLNVQREN#GTISRY VGGQEHFAH*LLILRDTKTYMLAFDVNDEKNWGLSVYADKPETTKEQLGEFY EALDCLRIPKSDVVYTDWKKDKCEPLEKQHEKERKQEEGES

Alpha-2-acid glycoprotein:

MALSWVLTVLSLLPLLEAQIPL*CANLVPVPITN#ATLDRITGKWFYIASAFRNEEY N#KSVQEIQATFFYFTPN#KTEDTIFLREYQTRQNQCFYN#SSYLNVQREN#GTVSR YEGGREHVA*HLLFLRDTKTLMFGSYLDDEKNWGLSFYADKPTTKEQLGEFYE ALDCLCIPRSDVMYTDWKKDKCEPLEKQHEKERKQEEGES

Fig. S3 N-glycosites of human AGP identified by trypsin (in red), by chylmotrypsin (in gray), by elastase (in italic), and by Gluc (underlined with black line). N# denotes the N-linked glycosylation site.

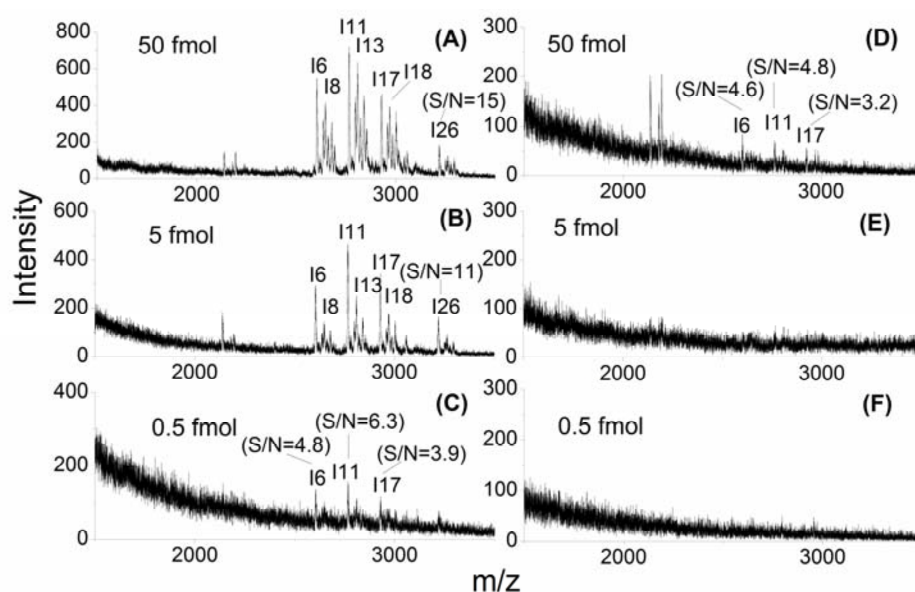

Fig. S4. Comparison of MALDI-TOF MS spectra of human IgG digest of (A) 50 fmol (0.5 μL), (B) 5 fmol (0.5 μL), (C) 0.5 fmol (0.5 μL), treated by Fe_3O_4@SiO_2@PEG-Maltose MNPs, and (D) 50 fmol (0.5 μL), (E) 5 fmol (0.5 μL), F) 0.5 fmol (0.5 μL), treated by silica-based click Maltose, respectively.

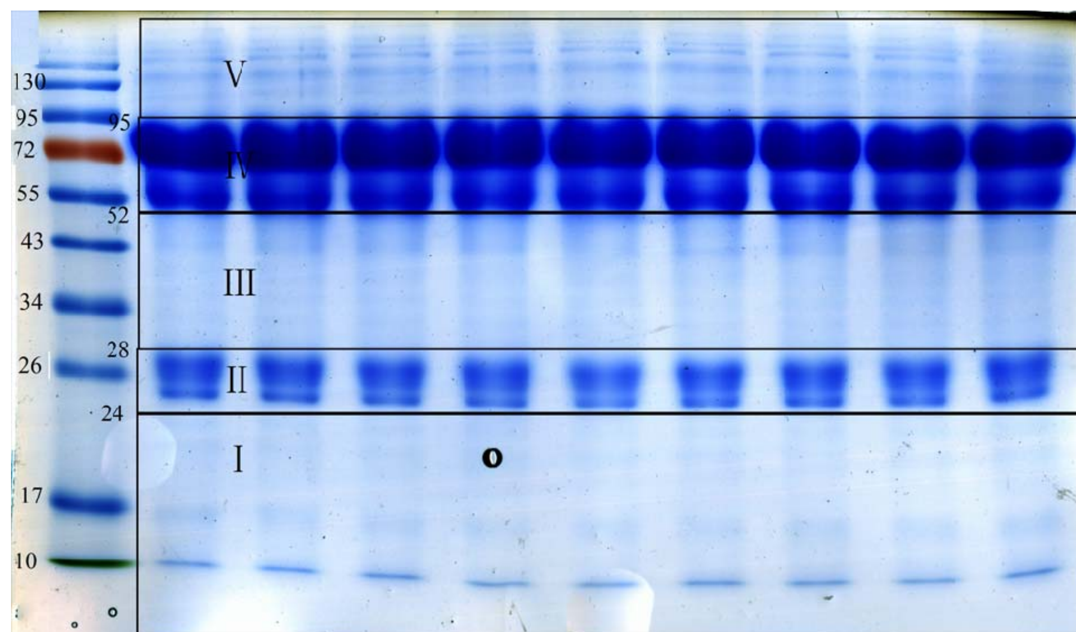

Fig. S5 Analysis of human plasma by SDS-PAGE. (Protein gel pieces were cut into five parts : Ⅰ(0- 24 KDa), Ⅱ(24- 28 KDa), Ⅲ(28- 52 KDa), Ⅳ(52- 95 KDa), and Ⅴ(95- KDa))

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

Peak number	Observed *m/z*	Glycan composition	Amino acid sequence
I1	2400.1	[Hex]3[HexNAc]3[Fuc]1	EEQFN#STFR
I2	2432.0	[Hex]3[HexNAc]3[Fuc]1	EEQYN#STYR
I3	2456.1	[Hex]3[HexNAc]4	EEQFN#STFR
I4	2488.1	[Hex]3[HexNAc]4	EEQYN#STYR
I5	2561.5	[Hex]4[HexNAc]3[Fuc]1	EEQFN#STFR
I6	2603.2	[Hex]3[HexNAc]4[Fuc]1	EEQFN#STFR
I7	2618.1	[Hex]4[HexNAc]4	EEQFN#STFR
I8	2635.6	[Hex]3[HexNAc]4[Fuc]1	EEQYN#STYR
I9	2674.4	[Hex]3[HexNAc]5	EEQFN#STYR
I10	2691.0	[Hex]3[HexNAc]5	EEQYN#STYR
I11	2764.6	[Hex]4[HexNAc]4[Fuc]1	EEQFN#STFR
I12	2780.1	[Hex]5[HexNAc]4	EEQFN#STFR
I13	2797.4	[Hex]4[HexNAc]4[Fuc]1	EEQYN#STYR
I14	2821.3	[Hex]4[HexNAc]5	EEQFN#STFR
I15	2838.1	[Hex]3[HexNAc]5[Fuc]1	EEQYN#STYR
I16	2853.3	[Hex]4[HexNAc]5	EEQYN#STYR
I17	2926.9	[Hex]5[HexNAc]4[Fuc]1	EEQFN#STFR
I18	2960.1	[Hex]5[HexNAc]4[Fuc]1	EEQYN#STYR
I19	2968.6	[Hex]4[HexNAc]5[Fuc]1	EEQFN#STFR
I20	2983.2	[Hex]5[HexNAc]5	EEQFN#STFR
I21	3000.6	[Hex]4[HexNAc]5[Fuc]1	EEQYN#STYR
I22	3015.1	[Hex]5[HexNAc]5	EEQYN#STYR
I23	3057.1	[Hex]4[HexNAc]4[Fuc]1[NeuAc]1	EEQFN#STFR
I24	3129.4	[Hex]5[HexNAc]5[Fuc]1	EEQFN#STFR
I25	3161.3	[Hex]5[HexNAc]5[Fuc]1	EEQYN#STYR
I26	3219.1	[Hex]5[HexNAc]4[Fuc]1[NeuAc]1	EEQFN#STFR
I27	3250.9	[Hex]5[HexNAc]4[Fuc]1[NeuAc]1	EEQYN#STYR

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

Peak number	Observed *m/z*	Glycan composition	Amino acid sequence
H1	1547.2	[Hex]2[HexNAc]1	PN#ATDTIPLVR
H2	1636.3	[Hex]2[HexNAc]1	SPN#ATDTIPLVR
H3	1844.1	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	SPN#ATDTIPLVR
H4	2321.5	[Hex]2[HexNAc]2	MGN#ITPLTGTQGQIR
H5	2438.2	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	SILLDN#TTSFR

H6	2509.0	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	ASILLDN#TTSFR
H7	2543.2	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	SSPN#ATDTIPLVR
H8	2612.6	[Hex]3[HexNAc]2[Xyl]1	MGN#ITPLTGTQGQIR
H9	2802.4	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	LFSSPN#ATDTIPLVR
H10	2850.8	[HexNAc]1[Fuc]1	GLIQSDQELFSSPN#ATDTIPLVR
H11	3061.3	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	QSDQELFSSPN#ATDTIPLVR
H12	3323.1	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	QLTPTFYDNSCPN#VSNIVR
H13	3355.2	[Hex]2[HexNAc]2[Fuc]1[Xyl]1	SFAN#STQTFFNAFVEAMDR
H14	3674.0	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	GLIQSDQELFSSPN#ATDTIPLVR
H15	3750.5	[Hex]3[HexNAc]2[Xyl]1	LHFHDCFVNGCDASILLDN#TTSFR
H16	3896.1	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	LHFHDCFVNGCDASILLDN#TTSFR
H17	4059.0	[Hex]3[HexNAc]2[Xyl]1	QLTPTFYDNSC(AAVESACPR)PN#V SNIVR-H_2O
H18	4223.9	[Hex]3[HexNAc]2[Fuc]1[Xyl]1	QLTPTFYDNSC(AAVESACPR)PN#V SNIVR
H19	4986.2	[Hex]2[HexNAc]2[Fuc]1[Xyl]1 [Hex]2[HexNAc]2[Fuc]1[Xyl]1	LYN#FSNTGLPDPTLN#TTYLQTLR

Table S3. List of identified glycoproteins from 15 μL human plasma captured by hybrid Fe_3O_4@SiO_2@PEG-Maltose MNPs, N# denotes the N-linked glycosylation site.

No	Protein	Description	Peptide sequence
1	IPI00792863	EDEM1 5 kDa protein	GNSSEFQKAVKLVINTVSFDK
2	IPI00387110	Ig kappa chain V-II region MIL	FSGSGSGTNFTLK
3	IPI00019399	SAA4 Serum amyloid A-4 protein	VYLQGLIDCYLFGNSSTVLEDSK
4	IPI00022731	APOC4 Apolipoprotein C-IV	ELLETVVNR
5	IPI00178926	IGJ Immunoglobulin J chain	ENISDPTSPLR IIVPLNNRENISDPTSPLR
6	IPI00013179	PTGDS Prostaglandin-H2 D-isomerase	WFSAGLASNSSWLR SVVAPATDGGLNLTSTFLR
7	IPI00006662	APOD Apolipoprotein D	CIQANYSLMENGK ADGTVNQIEGEATPVNLTEPAK ADGTVNQIEGEATPVNLTEPAKLEVK
8	IPI00030739	APOM Apolipoprotein M	TELFSSSCPGGIMLNETGQGYQR
9	IPI00022429	ORM1 Alpha-1-acid glycoprotein 1	ENGTISR NEEYNK QDQCIYNTTYLNVQR
10	IPI00020091.	ORM2 Alpha-1-acid	QNQCFYNSSYLNVQR

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		glycoprotein 2	SVQEIQATFFYFTPNKTEDTIFLR
11	IPI00022391	APCS Serum amyloid P-component	ESVTDHVNLITPLEKPLQNFTLCFR
12	IPI00022392	C1QA Complement C1q subcomponent subunit A	NPPMGGNVVIFDTVITNQEEPYQNHSGR RNPPMGGNVVIFDTVITNQEEPYQNHSGR RNPPMGGNVVIFDTVITNQEEPYQNHSG
13	IPI00025862	C4BPB Isoform 1 of C4b-binding protein beta chain	LGHCPDPVLVNGEFSSSGPVNVSDK EWDNTTTECR
14	IPI000006154	CFHR2 Isoform Long of Complement factor H-related protein 2	LQNNENNISCVER
15	IPI00431645	HP HP protein	MVSHHNLTTGATLINEQWLLTTAK
16	IPI00018305	IGFBP3 Insulin-like growth factor-binding protein 3	AYLLPAPPAPGNASESEEDR GLCVNASAVSR
17	IPI00293925	FCN3 Isoform 1 of Ficolin-3	VELEDFNGNR
18	IPI00166729	AZGP1 Zinc-alpha-2-glycoprotein	DIVEYYNDSNGSHVLQGR FGCEIENNR
19	IPI00022417	LRG1 Leucine-rich alpha-2-glycoprotein	LPPGLLANFTLLR AGLQAFFQVQECNK MFSQNDTR
20	IPI00027507	CFHR3 Complement factor H-related protein 3	FVQGNSTEVACHPGYGLPK
21	IPI00020986	LUM Lumican	LGSFEGLVNLTFIHLQHNR AFENVTDLQWLILDHNLLENSK
22	IPI00477597	HPR Isoform 1 of Haptoglobin-related protein	NLFLNHSENATAK
23	IPI00298828	APOH Beta-2-glycoprotein 1	DTAVFECLPQHAMFGNDTITCTTHGNWTK VYKPSAGNNSLYR LGNWSAMPSCK PSAGNNSLYR
24	IPI00218732	PON1 Serum paraoxonase/arylesterase 1	HANWTLTPLK VTQVVYAENGTVLQGSTVASVYK VVAEGFDFANGINISPDGK SLDFNTLVDNISVDPETGDLWVGCHPNGMK
25	IPI00479708	IGHM Full-length cDNA clone CS0DD006YL02 of Neuroblastoma of	STGKPTLYNVSLVMSDTAGTCY

26	IPI00385264	Homo sapiens Ig mu heavy chain disease protein	THTNISESHPNATFSAVGEASICEDDWDSGER
27	IPI00027482	SERPINA6 Corticosteroid-binding globulin	AQLLQGLGFNLTER AVLQLNEEGVDTAGSTGVTLNLTSKPIILR
28	IPI00009030	LAMP2 Isoform LAMP-2A of Lysosome-associated membrane glycoprotein 2	IAVQFGPGFSWIANFTK
29	IPI00006114	SERPINF1 Pigment epithelium-derived factor	VTQNLTLIEESLTSEFIHDIDR
30	IPI00292946	SERPINA7 Thyroxine-binding globulin	TLYETEVFSTDFSNISAAK
31	IPI00399007	IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	NQVSLTCLVK
32	IPI00553177	SERPINA1 Isoform 1 of Alpha-1-antitrypsin	YLGNATAIFFLPDEGK ADTHDEILEGLNFNLTEIPEAQIHEGFQELLR QLAHQSNSTNIFFSPVSIATAFAMLSLGTK
33	IPI00641737	HP Haptoglobin	VVLHPNYSQVDIGLIK
34	IIPI00022431	AHSG cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotei n	VCQDCPLLAPLNDTR AALAAFNAQNNGSNFQLEEISR
35	IPI00328609	SERPINA4 Kallistatin	SQILEGLGFNLTELSESDVHR
36	IPI00215894	KNG1 Isoform LMW of Kininogen-1	ITYSIVQTNCSK
37	IPI00550991	SERPINA3 cDNA FLJ35730 fis, clone TESTI2003131, highly similar to ALPHA-1-ANTICHY MOTRYPSIN	YTGNASALFILPDQDK FNLTETSEAEIHQSFQHLLR NVIFSPLSISTALAFLSLGAHNTTLTEILK TLNQSSDELQLSMGNAMFVK
38	IPI00785084	IGHG1;IGHV4-31;LOC100290146 IGH@ protein	EEQYNSTYR
39	IPI00426051	IGHG2 Putative uncharacterized protein	EEQFNSTFR

		DKFZp686C15213	
40	IPI00896380	IGHM Isoform 2 of Ig mu chain C region	NNSDISSTR YKNNSDISSTR THTNISESHPNATFSAVGEASICEDDWNSGER GLTFQQNASSMCVPDQDTAIR
41	IPI00022488	HPX Hemopexin	SWPAVGNCSSALR CSDGWSFDATTLDDNGTMLFFK ALPQPQNVTSLLGCTH NGTGHGNSTHHGPEYMR GHGHRNGTGHGNSTHHGPEYMR
42	IPI00032179	SERPINC1 Antithrombin-III	WVSNK LGACNDTLQQLMEVFK SLTFNETYQDISELVYGAK
43	IPI00291262	CLU Isoform 1 of Clusterin	MLNTSSLLEQLNEQFNWVSR ELPGVCNETMMALWEECKPCLK MLNTSSLLEQLNEQFNWVS KKEDALNETR HNSTGCLR EDALNETR KEDALNETR LANLTQGEDQYYLR
44	IPI00930442	IGHG4 Putative uncharacterized protein DKFZp686M24218	EEQFNSTYR EEQFNSTY
45	IPI00784758	LOC100126583;IGHA2 Putative uncharacterized protein DKFZp686M08189	TPLTANITK
46	IPI00032220	AGT Angiotensinogen	VYIHPFHLVIHNESTCEQLAK
47	IPI00386879	IGHA1 cDNA FLJ14473 fis, clone MAMMA1001080, highly similar to Homo sapiens SNC73 protein (SNC73) mRNA	LAGKPTHVNVSVVMAEVDGTCY
48	IPI00009793	C1RL Complement C1r subcomponent-like protein	PVTPIAQNQTTLGSSR
49	IPI00383164	IGHA1 SNC66 protein	LSLHRPALEDLLLGSEANLTCTLTGLR
50	IPI00642017	LOC100126583;IGHA2 Putative uncharacterized protein DKFZp686C02218 (Fragment)	PALEDLLLGSEANLTCTLTGLR

51	IPI00894384	LOC100126583;IGHA2 Putative uncharacterized protein DKFZp686O16217 (Fragment)	HYTNSSQDVTVPCR PALEDLLLGSEANLTCTLTGL
52	IPI00298971	VTN Vitronectin	NNATVHEQVGGPSLTSDLQAQSK NISDGFDGIPDNVDAALALPAHSYSGR
53	IPI00291866	SERPING1 Plasma protease C1 inhibitor	VGQLQLSHNLSLVILVPQNLK DTFVNASR VLSNNSDANLELINTWVAK
54	IPI00384280	PCYOX1 Prenylcysteine oxidase 1	LLHALGGDDFLGMLNR
55	IPI00418153	IGHM Putative uncharacterized protein DKFZp686I15212	EEQYNSTFR
56	IPI00009865	KRT10 Keratin, type I cytoskeletal 10	TIDDLKNQILNLTTDNANILLQIDNAR NVSTGDVNVEMNAAPGVDLTQLLNNMR
57	IPI00292950	SERPIND1 Serpin peptidase inhibitor, clade D (Heparin cofactor), member 1	NLSMPLLPADFHK DFVNASSK
58	IPI00022371	HRG Histidine-rich glycoprotein	VIDFNCTTSSVSSALANTK
59	IPI00479116	CPN2 Carboxypeptidase N subunit 2	LYLGSNNLTALHPALFQNLSK LEDLEVTGSSFLNLSTNIFSNLTSLGK
60	IPI00218413	BTD Biotinidase	NPVGLIGAENATGETDPSHSK DVQIIVFPEDGIHGFNFTR WNVNAPPTFHSEMMYDNFTLVPVWGK
61	IPI00019359	KRT9 Keratin, type I cytoskeletal 9	NYSPYYNTIDDLKDQIVDLTVGNNK
62	IPI00163207	PGLYRP2 Isoform 1 of N-acetylmuramoyl-L-alanine amidase	GFGVAIVGNYTAALPTEAALR LEPVHLQLQCMSQEQLAQVAANATK
63	IPI00022395	C9 Complement component C9	AVNITSENLIDDVVSLIR
64	IPI00021304	KRT2 Keratin, type II cytoskeletal 2 epidermal	FGGFGGPGGVGGLGGPGGFGPGGYPGGIHEVSVNQSLLQPLNVK MSGDLSSNVTVSVTSSTISSNVASK
65	IPI00023673	LGALS3BP Galectin-3-binding protein	ALGFENATQALGR AAIPSALDTNSSK
66	IPI00011252	C8A Complement	GGSSGWSGGLAQNR

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		component C8 alpha chain	
67	IPI00043716	LCORL Isoform 1 of Ligand-dependent nuclear receptor corepressor-like protein	NSSKPV
68	IPI00291867	CFI Complement factor I	LISNCSK FLNNGTCTAEGK
69	IPI00021727	C4BPA C4b-binding protein alpha chain	FSLLGHASISCTVENETIGVWRPSPPTCEK
70	IPI00019943	AFM Afamin	DIENFNSTQK YAEDKFNETTEK
71	IPI00019568	F2 Prothrombin (Fragment)	GHVNITR YPHKPEINSTTHPGADLQENFCR NFTENDLLVR WVLTAAHCLLYPPWDKNFTENDLLVR
72	IPI00029193	HGFAC Hepatocyte growth factor activator	DSVSVVLGQHFFNR
73	IPI00032328	KNG1 Isoform HMW of Kininogen-1	LNAENNATFYFK YNSQNQSNNQFVLYR
74	IPI00654888	KLKB1 Plasma kallikrein	GVNFNVSK IVGGTNSSWGEWPWQVSLQVK IYSGILNLSDITK IYPGVDFGGEELNVTFVK LQAPLNYTEFQKPICLPSK
75	IPI00025864	BCHE Butyrylcholinesterase, isoform CRA_b	YGNPNETQNNSTSWPVFK
76	IPI00969516	LOC100293534 similar to complement component 4B (Childo blood group), partial	GLNVTLSSTGR
77	IPI00017696	C1S Complement C1s subcomponent	NCGVNCSGDVFTALIGEIASPNYPKPYPENSR
78	IPI00022463	TF Serotransferrin	CGLVPVLAENYNK QQQHLFGSNVTDCSGNFCLFR
79	IPI00298860	LTF cDNA FLJ78440, highly similar to Human lactoferrin	TAGWNVPIGTLRPFLNWTGPPEPIEAAVAR
80	IPI00292218	MST1 Hepatocyte growth factor-like protein	GTANTTTAGVPCQR
81	IPI00290283	MASP1 Isoform 2 of Mannan-binding lectin	SGAVNSSAAR

		serine protease 1	
82	IPI00296165	C1R cDNA FLJ54471, highly similar to Complement C1r subcomponent	EHEAQSNASLDVFLGHTNVEELMK
83	IPI00004573	PIGR Polymeric immunoglobulin receptor	LSLLEEPGNGTFTVILNQLTSR
84	IPI00299503	GPLD1 Isoform 1 of Phosphatidylinositol-glycan-specific phospholipase D	LNVEAANWTVR LGTSLSSGHVLMNGTLK
85	IPI00296608	C7 Complement component C7	NYTLTGR INNDFNYEFYNSTWSYVK
86	IPI00028413	ITIH3 Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H3	NAHGEEKENLTAR
87	IPI00218192	ITIH4 Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H4	AFITNFSMIIDGMTYPGIIK LPTQNITFQTESSVAEQEAEFQSPK
88	IPI00292530	ITIH1 Inter-alpha-trypsin inhibitor heavy chain H1	ANLSSQALQMSLDYGFVTPLTSMSIR ICDLLVANNHFAHFFAPQNLTNMNK DKICDLLVANNHFAHFFAPQNLTNMNK
89	IPI00305461	ITIH2 Inter-alpha (Globulin) inhibitor H2, isoform CRA_a	GAFISNFSMTVDGK
90	IPI00005031	PCDH11X Isoform 7 of Protocadherin-11 X-linked	YSIVGGNTRDLFAIDQETGNITLMEK
91	IPI00025753	DSG1 Desmoglein-1	TGEINITSIVDR
92	IPI00017601	CP Ceruloplasmin	ELHHLQEQNVSNAFLDK ENLTAPGSDSAVFFEQGTTR EHEGAIYPDNTTDFQR DVDKEFYLFPTVFDENESLLLEDNIR
93	IPI00947496	124 kDa protein	LGSYPVGGNVSFECEDGFILR
94	IPI00297931	SYNRG Isoform 1 of Synergin gamma	MPPWIYNESLVPDAYK
95	IPI00019591	CFB cDNA FLJ55673, highly similar to Complement factor B	TMFPNLTDVR IVLDPSGSMNIYLVLDGSDSIGASNFTGAK
96	IPI00029739.	CFH Isoform 1 of	SPDVINGSPISQK

		Complement factor H	IPCSQPPQIEHGTINSSR MDGASNVTCINSR ISEENETTCYMGK WDPEVNCSMAQIQLCPPPPQIPNSHNMTTTLNYR
97	IPI00162735	ATRN Isoform 2 of Attractin	IDSTGNVTNELR
98	IPI00027235	ATRN Isoform 1 of Attractin	VFHIHNESWVLLTPK
99	IPI00478003	A2M Alpha-2-macroglobulin	GCVLLSYLNETVTVSASLESVR SLGNVNFTVSAEALESQELCGTEVPSVPEHGR GNEANYYSNATTDEHGLVQFSINTTNVMGTSLTVR
100	IPI00783987	C3 Complement C3 (Fragment)	TVLTPATNHMGNVTFTIPANR
101	IPI00418163	C4B;C4A complement component 4B preproprotein	FSDGLESNSSTQFEVK
102	IPI00654875	C4B Complement C4-B	NLTVSVHVSPVEGLCLAGGGGLAQQVLVPAGSAR
103	IPI00032258	C4A Complement C4-A	NTTCQDLQIEVTVK
104	IPI00022418	FN1 Isoform 1 of Fibronectin	LDAPTNLQFVNETDSTVLVR DQCIVDDITYNVNDTFHK LDAPTNLQFVNETDSTVLV
105	IPI00217052	NAV2 Isoform 1 of Neuron navigator 2	NRSQMIENIDACLNFLAAK
106	IPI00022229	APOB Apolipoprotein B-100	FEVDSPVYNATWSASLK DFHSEYIVSASNFTSQLSSQVEQFLHR FNSSYLQGTNQITGR VNQNLVYESGSLNFSK ELCTISHIFIPAMGNITYDFSFK IQSPLFTLDANADIGNGTTSANEAGIAASITAK FVEGSHNSTVSLTTK