Supporting Information

Specific enrichment and glycosylation discrepancy profiling of cellular exosomes using a dual-affinity probe

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Experiment section

Chemicals. 20×TBS and 1×PBS were purchased from Solarbio. Anti-TSG101 polyclonal antibody and goat anti-rabbit IgG (HRP conjugated) were purchased from MultiSciences (Lianke) Biotech. Sequencing grade trypsin was purchased from Promega. 1,1'-dioctadecyl-3,3,3',3'tetramethylindocarbocyanine perchlorate (DiI) were purchased from Meilun Biotechnology Company. Phosphotungstic acid hydrate was purchased from Alfa Aesar. Dithiothreitol (DTT), iodoacetamide (IAA), urea, thiourea, phenylmethanesulfony fluoride (PMSF), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. Titanium (Ⅳ) butoxide (TBOT) and glutathione (GSH) were purchased from Adamas beta. Iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ethylene glycol, sodium acetate anhydrous and concentrated ammonia solution (28 wt%) were purchased from Guoyao Chemical Reagent Company. Acetonitrile (ACN) was purchased from Merck. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Scientific.

Synthesis of Fe_3O_4@TiO_2-GSH. Generally speaking, $FeCl_3 \cdot 6H_2O$ and CH_3COONa were dispersed in 75 mL ethylene glycol to form Fe_3O_4 nanoparticles via the solvo-thermal reaction. The above magnetic core and 1.6 mL titanium (Ⅳ) butoxide was dispersed in 200 mL ethyl alcohol under the basic condition, keeping stirring violently at 45 ℃ for 24 h. Then Fe_3O_4@TiO_2 were synthesized and calcined in a muffle furnace at 400 ℃ for 2 h. Next, the materials were redispersed in 30 mL water which containing800 mg GSH. And the reaction was performed at room temperature for 20 h. Finally, Fe_3O_4@TiO_2-GSH was synthesized.

Cell culture. MCF-7 cells, MDA-MB-231 cells and Hela cells were cultured in petri dishes (100 mm diameter, 20 mm height) with 10% FBS, 89.5% DMEM and 0.5% penicillin-streptomycin and set in a humidified incubator with 5% CO_2 at 37 ℃. The above supernatant was instead by condition media without FBS since 80% bottom area of the petri dish was covered by cells. After incubation for 24 h, the late supernatant was collected to capture exosomes.

Preparation of exosomes samples originated from cells. The collected supernatants of different cells were filtered with filter (0.22 μm) to remove apoptotic blebs, cell debris, and cells. Then the filtrates were concentrated through an ultrafiltration tube (Merck Millipore, 100 kDa) and some proteins and peptides were removed together. Afterwards, the concentrate were dispersed in 1×TBS and stored at -80 ℃ for further use.

Exosomes isolation. First of all, 6.0 mg Fe_3O_4@TiO_2-GSH were incubated with 1 mL pre-treated cell samples at 4 ℃ for 10 min. Secondly, the supernatants were removed with the effect of magnet and the sediments were washed three times with PBS. Finally, the enriched exosomes were eluted by 0.4 M ammonia aqueous solution for 10 min at 4 ℃ and the solvents were substituted by PBS with ultrafiltration tubes to keep exosomes activity.

Exosomes dye. 40 μg purified exosomes secreted by MCF-7 cells was incubated in 1×TBS buffer containing 10 μM DiI and placed on the shaker at 37 ℃ for 15 min in the dark. After reacting with Fe_3O_4@TiO_2-GSH, the fluorescence intensity of supernatants and eluents were detected by fluorescence spectrometer (Hitachi, F-7000) at excitation and emission wavelengths of 545 nm and 570 nm to obtain the materials capture efficiency and exosomes

recovery. And the above steps were repeated three times in parallel to get an average result. In addition, the same method was used for the other two kinds of exosomes from Hela cells and MDA-MB-231 cells.

Exosomes lysis and digestion. Isolated exosomes were suspended in the urea lysis buffer containing 8 M urea, 2 M thiourea and 1 mM PMSF and ultrasonicated for 15 min. After centrifugation at 10,000 *g* for 5 min, the supernatant were substituted by 25 mM NH_4HCO_3 buffer using ultrafiltration tubes (3 kDa). Following by heating for 10 min at 90 ℃, the proteins were reduced by 10 mM DTT at 56 ℃ for 1 h and alkylated by 25 mM IAA in the dark at 37 ℃ for 0.5 h. Then trypsin was added in the samples (trypsin: protein=1:40, w/w), and incubated at 37 ℃ for 16 h. Finally, the peptides were collected after desalting and lyophilizing.

Enrichment of exosomal N-linked glycopeptides. Exosomes lysate were dispersed in 100 μL 90%ACN/3%TFA (v/v) solution and 800 μg Fe_3O_4@TiO_2-GSH were incubated in the mixture at 37 ℃ for 0.5 h to capture glycopeptides. After washing by the above loading buffer to remove impurities, the goal peptides were eluted by 50%ACN aqueous solution for 45 min. Finally, the eluents were then deglycosylated in the mixture of 49 μL NH_4HCO_3 (25 mM, pH = 7.8) and 1 μL PNGase F.

Nano-LC-MS/MS analysis. The solvent A (water containing 0.1% formic acid) and solvent B (ACN containing 0.1% formic acid) were prepared. The lyophilized eluent was dissolved with 10 μL solvent A. The captured peptides were separated by Nano-LC, and on-line electrospray tandem mass spectrometry was used to analyze them. The experiments

were performed on an EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an online nano-electrospray ion source. A 4 μL peptide sample was loaded on the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm × 2 cm) and separated on the analytical column (Acclaim PepMap C18, 75 μm × 25 cm) with a linear gradient, from 2% B to 40% B in 110 min. The column was re-equilibrated at initial conditions for 15 min with the column flow rate at 300 nL min^{-1} and column temperature at 40 °C. A data-dependent mode was adopted in the Orbitrap Fusion mass spectrometer to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350–1500) were gained in the Orbitrap with a mass resolution of 120,000 at m/z 200. The AGC target was set to 1,000,000 with maximum injection time at 50 ms. MS/MS acquisition was performed in the Orbitrap with a cycle time of 3 s. The threshold value of intensity was 50,000 and maximum injection time was 100 ms. The AGC target was set to 200,000 with the isolation window at 2 m/z.

Tandem mass spectra were extracted using Proteome Discoverer software (Thermo Fisher Scientific, version 1.4.0.288). The human UniProtKB/Swiss-Prot database (Release 2015-03-11, with 20199 sequences) was chosen as the database and Mascot (Matrix Science, London, UK; version 2.3.2) was used to analyze all MS/MS data. The enzyme was specified as trypsin. The parent ion mass tolerance and fragment ion mass tolerance were specified as 10.0 ppm and 50 mmu, respectively. Carbamidomethylation of cysteine was specified as the fixed modification. Meanwhile, variable modifications included

94 oxidation on methionine and deamidation on asparagine. The percolator algorithm was
95 employed to make sure that peptide level false discovery rates (FDRs) were lower than 1%.
96 The identified deamination sites were confirmed to be glycosylation sites only when they
97 were consistent with the N-glycosylation consensus sequence (n-!P-[S/T/C]).
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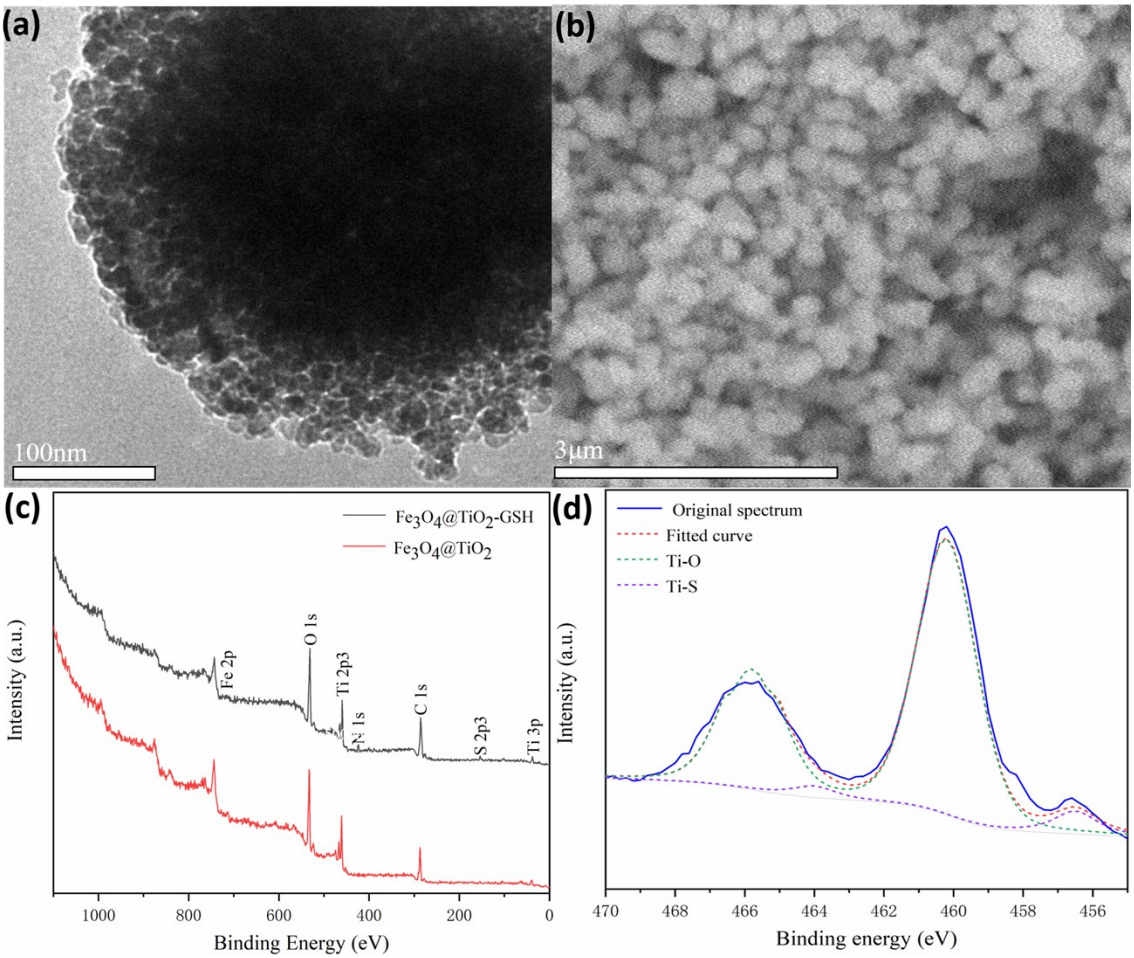
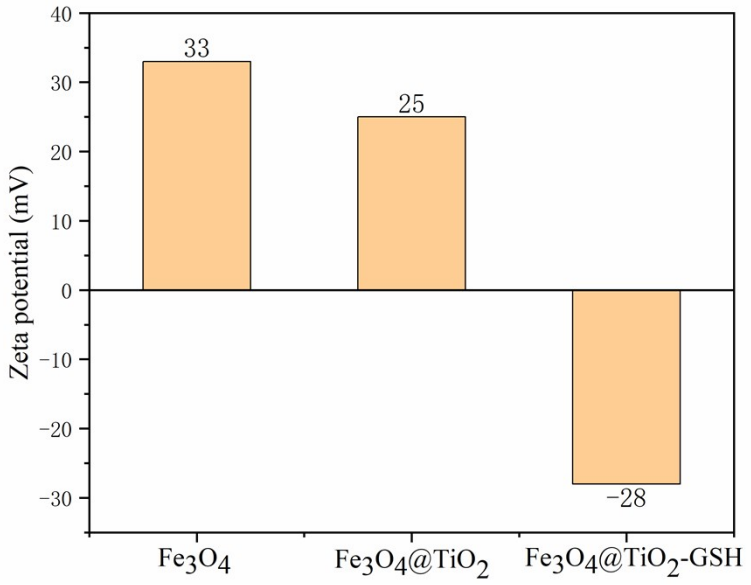


Figure S1. (a) TEM and (b) SEM image of Fe_3O_4@TiO_2-GSH; (c) X-ray photoelectron spectrum of Fe_3O_4@TiO_2 and Fe_3O_4@TiO_2-GSH; (d) High-resolution XPS spectrum of Ti 2p.



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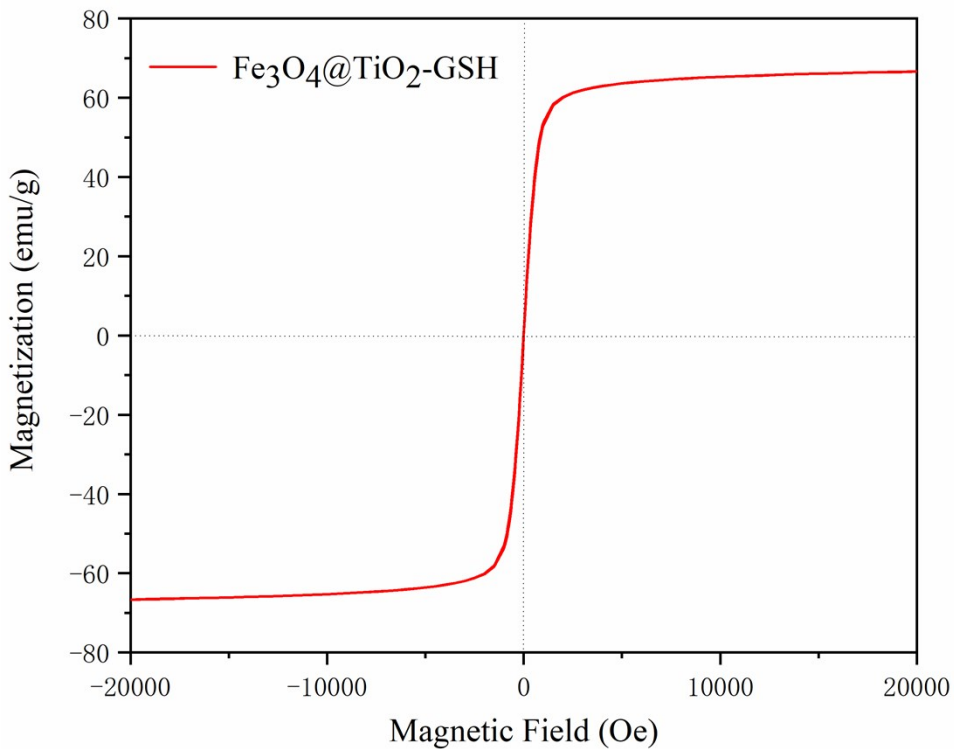
104 **Figure S2.** Zeta potential of Fe_3O_4 , Fe_3O_4@TiO_2 and Fe_3O_4@TiO_2-GSH.



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106 **Figure S3.** Magnetic hysteresis curves of Fe_3O_4@TiO_2-GSH.

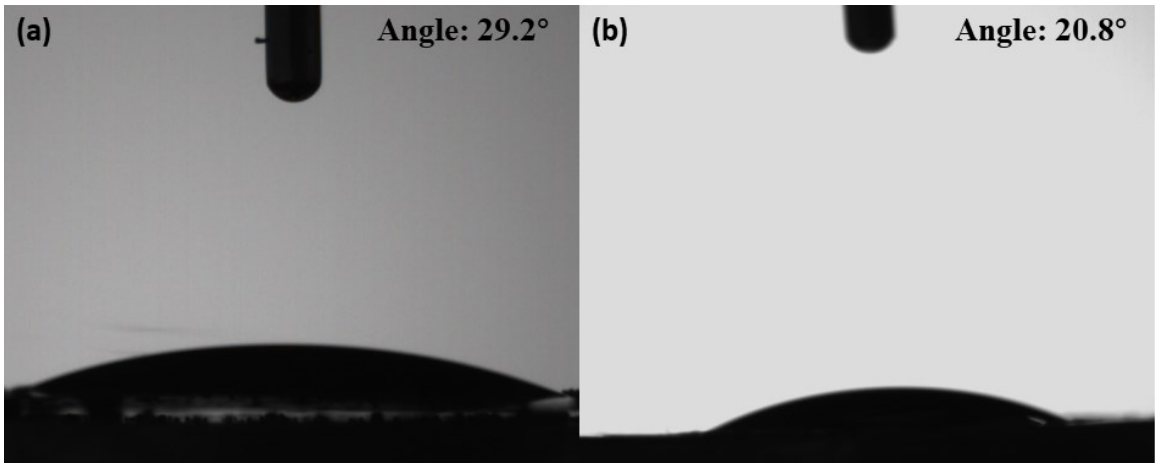


Figure S4. Contact angle of (a) Fe_3O_4@TiO_2 and (b) Fe_3O_4@TiO_2-GSH.

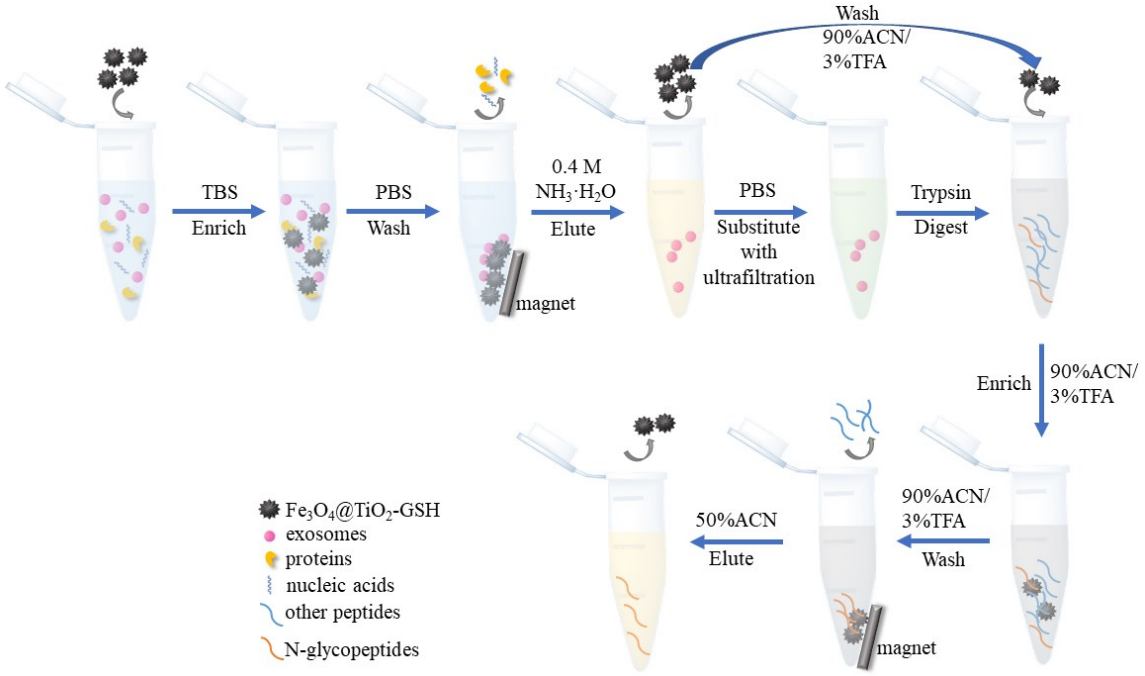


Figure S5. Workflow of exosomes and N-glycopeptides consecutive enrichment from cells culture medium using Fe_3O_4@TiO_2-GSH.

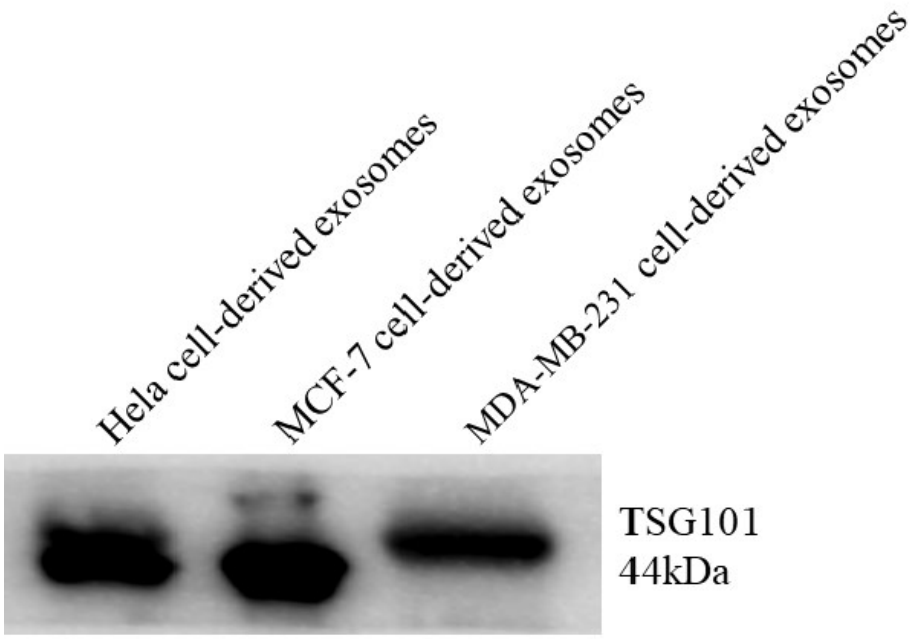


Figure S6. Western blot of TSG101 protein from Hela cells, MDA-MB-231 cells and MCF-7 cells derived exosomes isolated by Fe_3O_4@TiO_2-GSH.

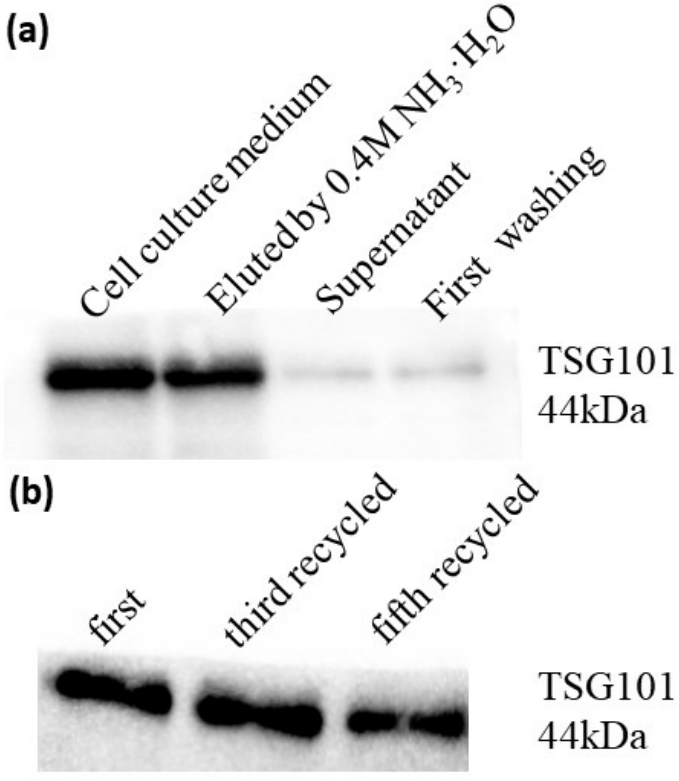


Figure S7. (a) Western blot results of TSG101 protein in isolated Hela cells-derived

exosomes during the enrichment process with Fe_3O_4@TiO_2-GSH; (b) Western blot results of TSG101 protein in isolated MCF-7 cell-derived exosomes with first / third / fifth recycled Fe_3O_4@TiO_2-GSH.

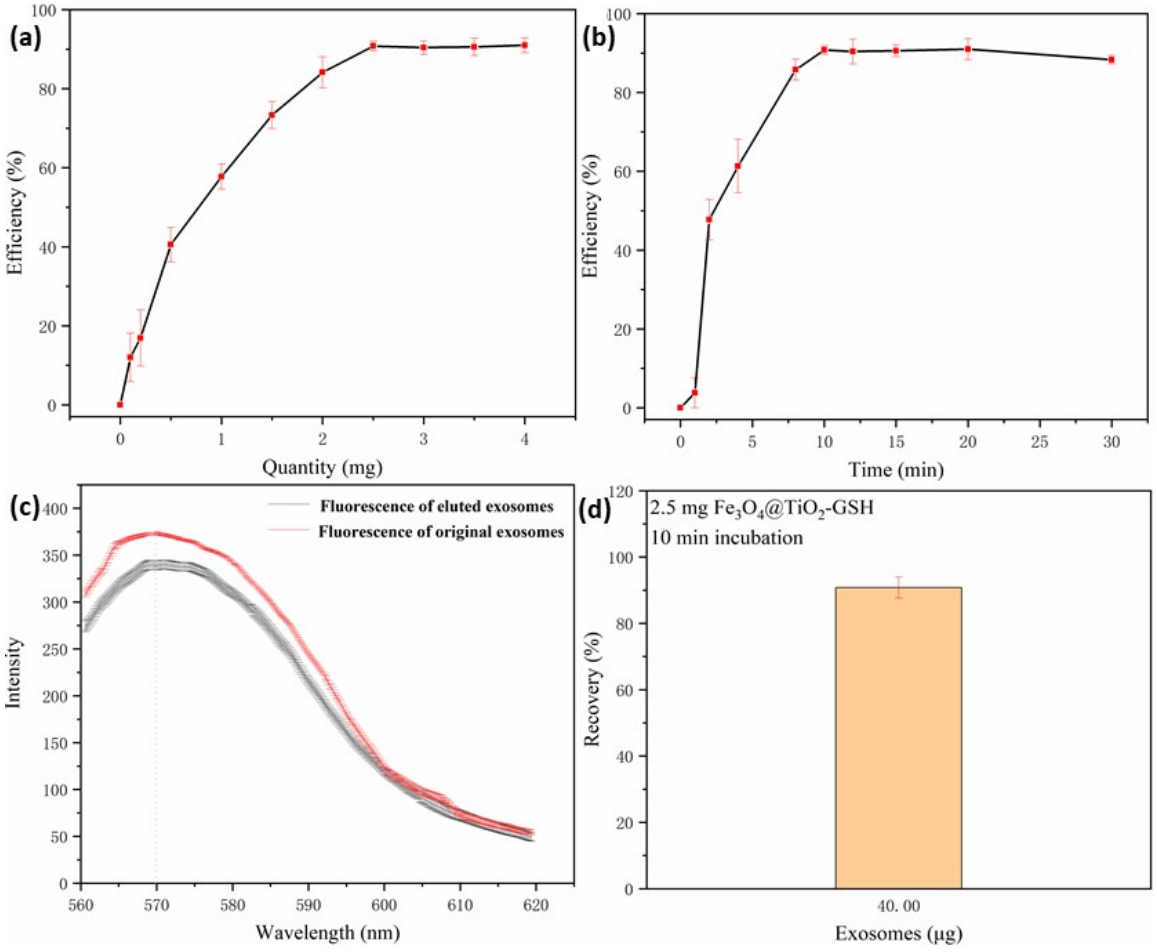
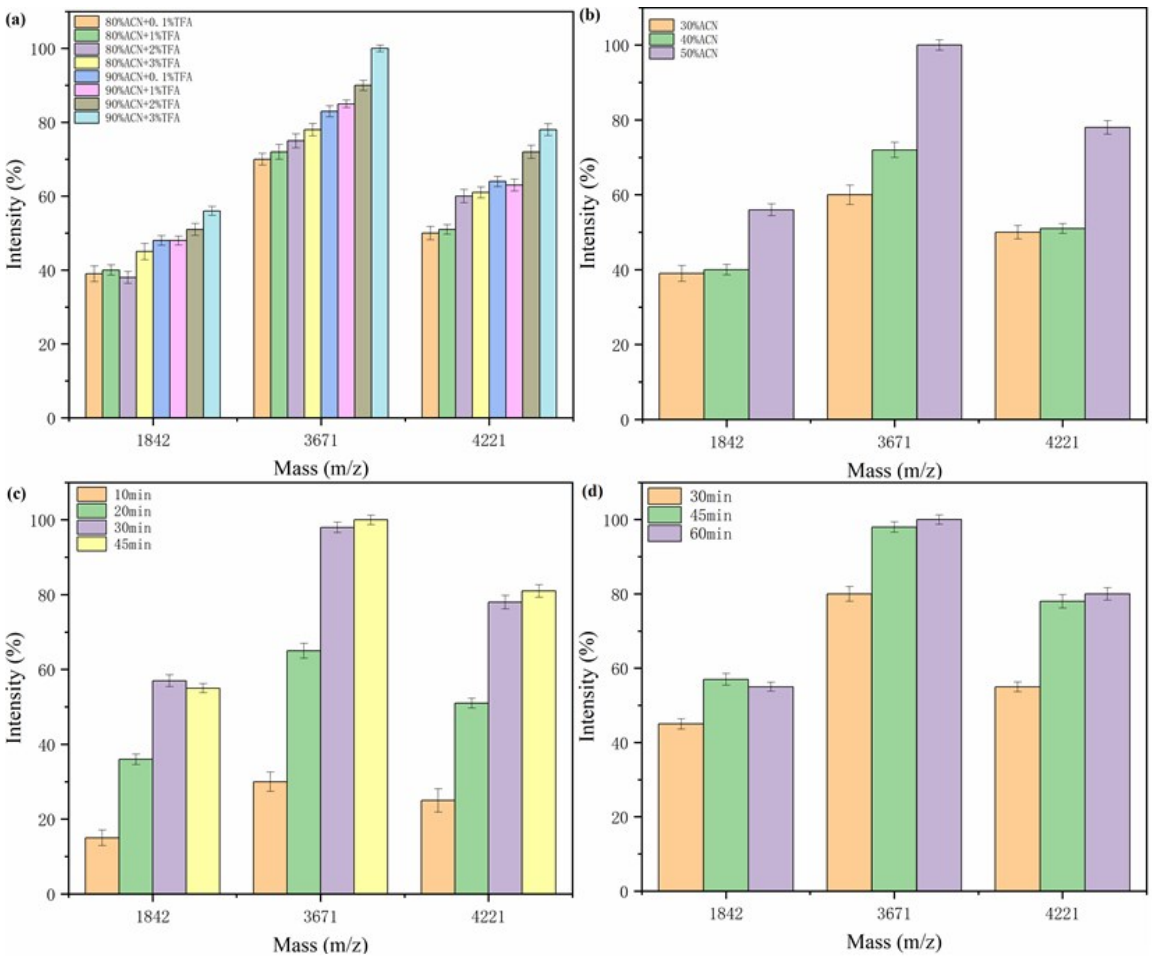


Figure S8. (a) Optimization of the quantity of Fe_3O_4@TiO_2-GSH; (b) Optimization of incubation time for exosome isolation; (c) Fluorescence curve of original purified exosomes and eluted exosomes from Fe_3O_4@TiO_2-GSH; (d) The recovery of exosomes isolated by Fe_3O_4@TiO_2-GSH under the optimal condition.



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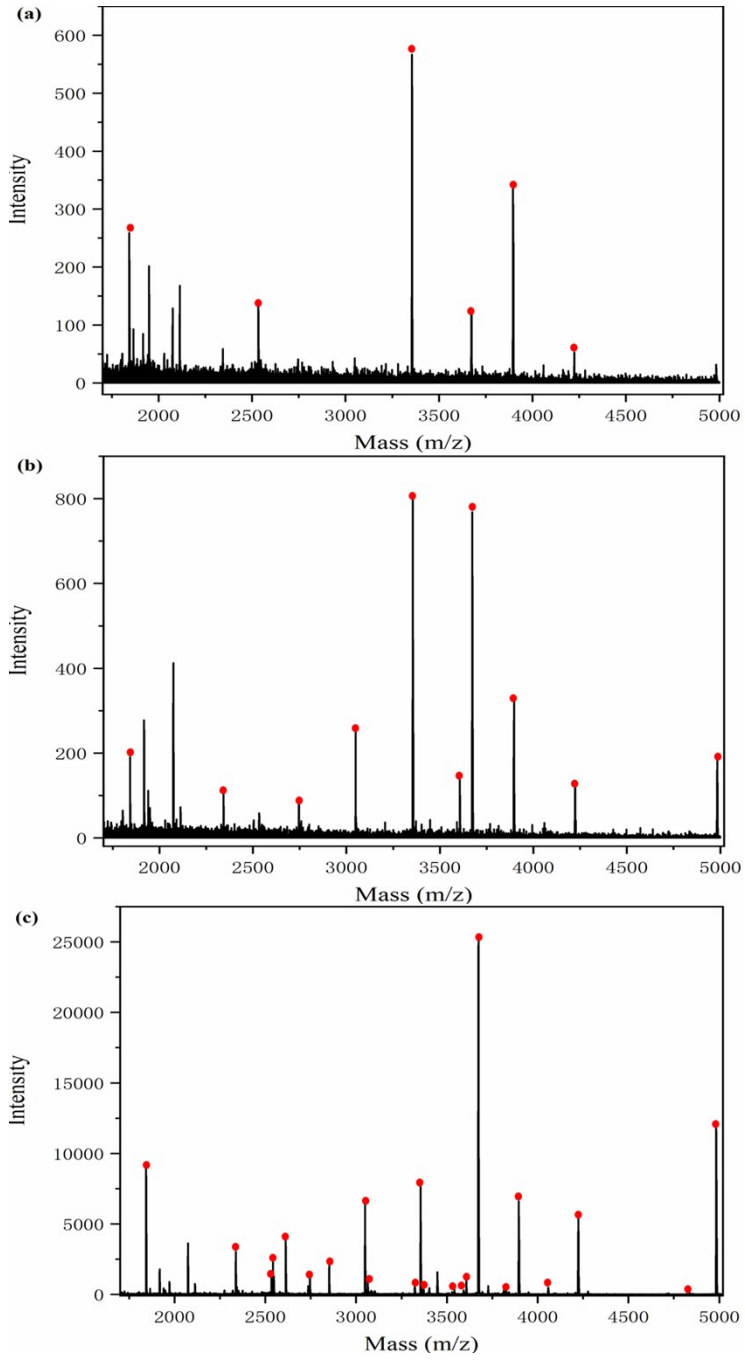
126 **Figure S9**. Optimization of (a) loading buffer, (b) elution buffer, (c) incubation time and

127 (d) elution time during the glycopeptides enrichment process with Fe_3O_4@TiO_2-GSH.



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129 **Figure S10**. MALDI-TOF mass spectra of tryptic digested HRP (100 fmol μL^{-1}): (a) before

130 enrichment; (b) after enrichment by Fe_3O_4@TiO_2; (c) after enrichment by Fe_3O_4@TiO_2-

131 GSH. Mass spectrometric peaks of N-glycopeptides are marked with red circles.

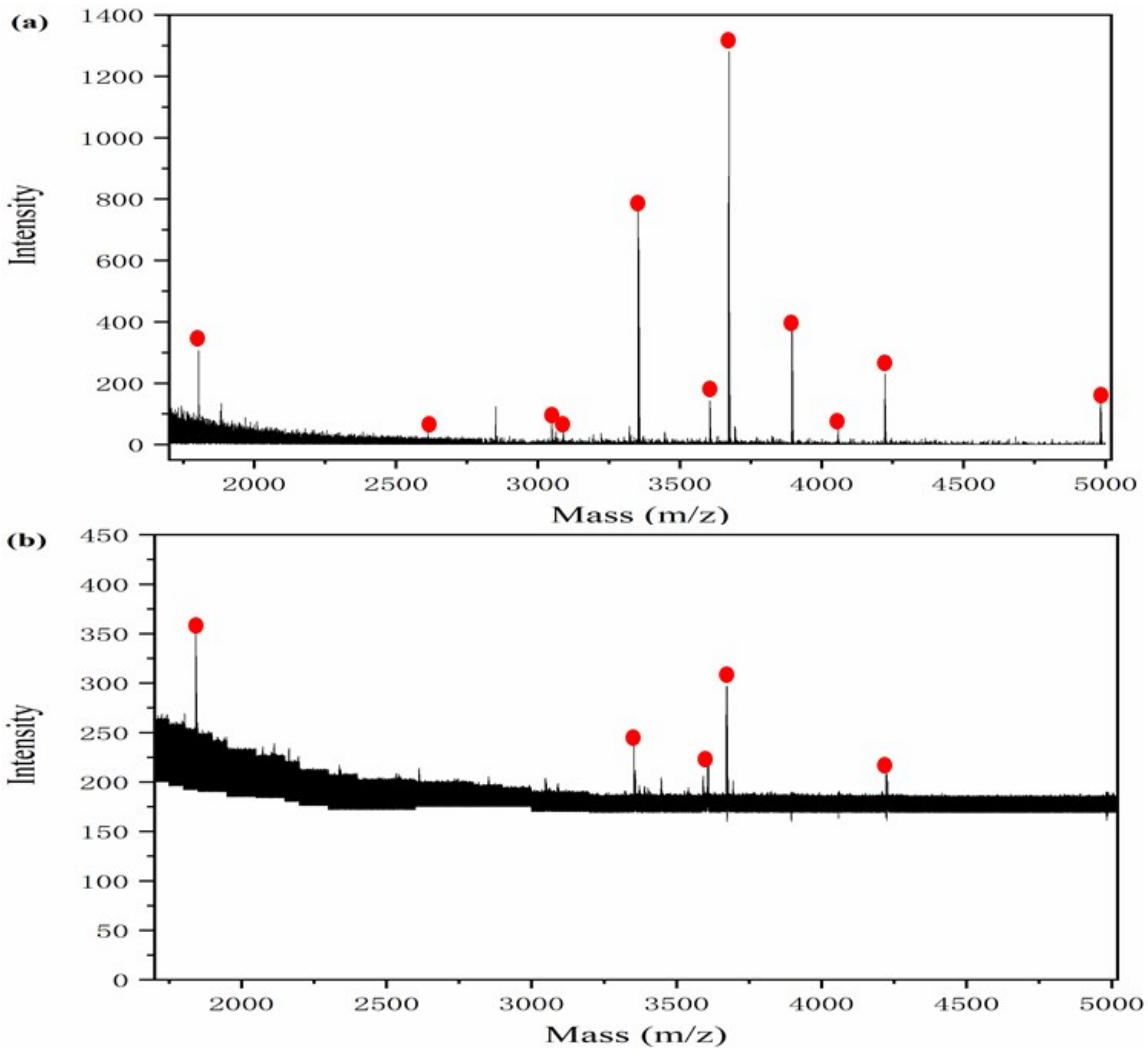
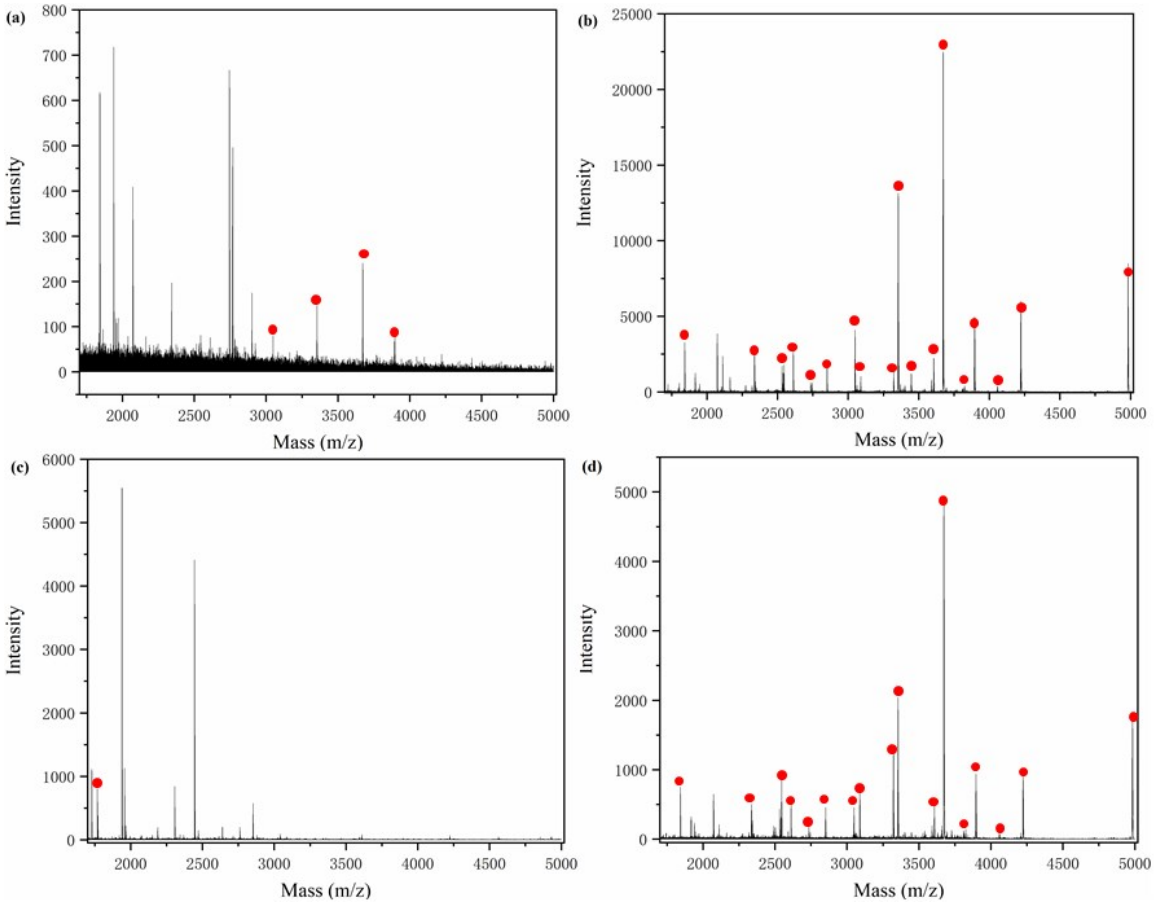


Figure S11. MALDI-TOF mass spectra of tryptic digested HRP after enrichment by Fe_3O_4@TiO_2-GSH with the concentration of: (a) 5 fmol μL^{-1} and (b) 0.5 fmol μL^{-1}. And mass spectrometric peaks of glycopeptides are marked with red circles.



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137 **Figure S12**. MALDI-TOF mass spectra for the glycopeptide enrichment from a mixture of

138 tryptic digests of HRP and BSA at a mass ratio of 1 : 10: (a) before and (b) after treatment

139 with Fe_3O_4@TiO_2-GSH; (c) before and (d) after treatment with Fe_3O_4@TiO_2-GSH at a

140 mass ratio of 1 : 100 (HRP : BSA), where glycopeptides are marked with red circles.

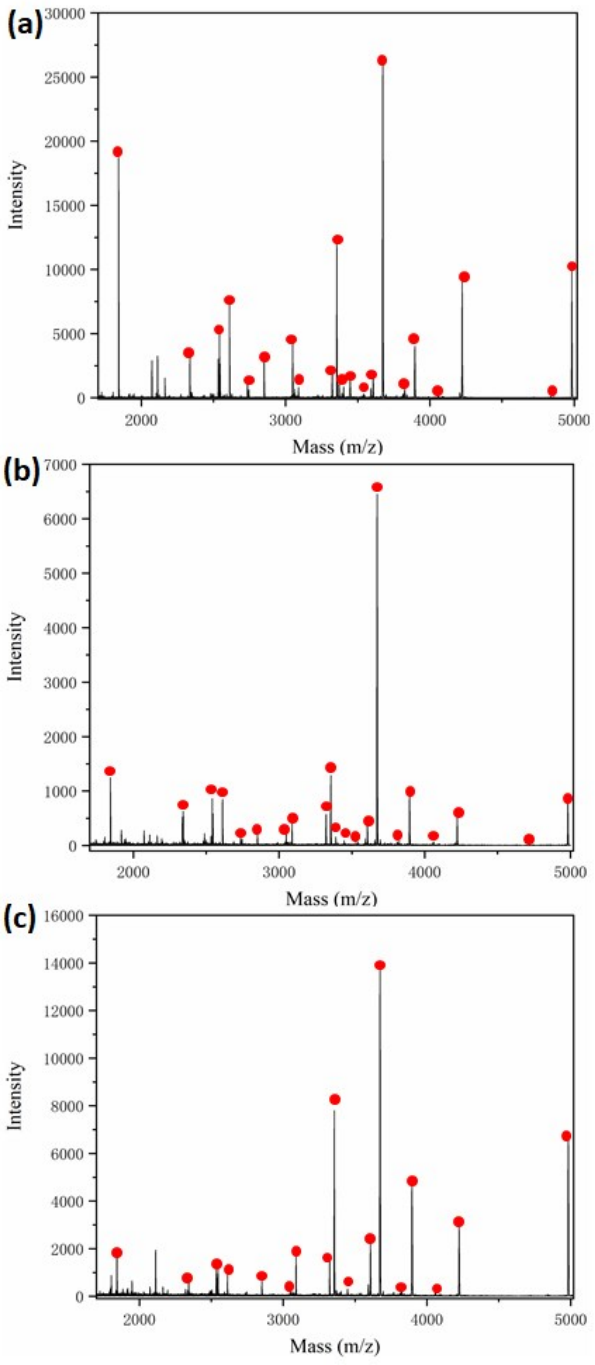
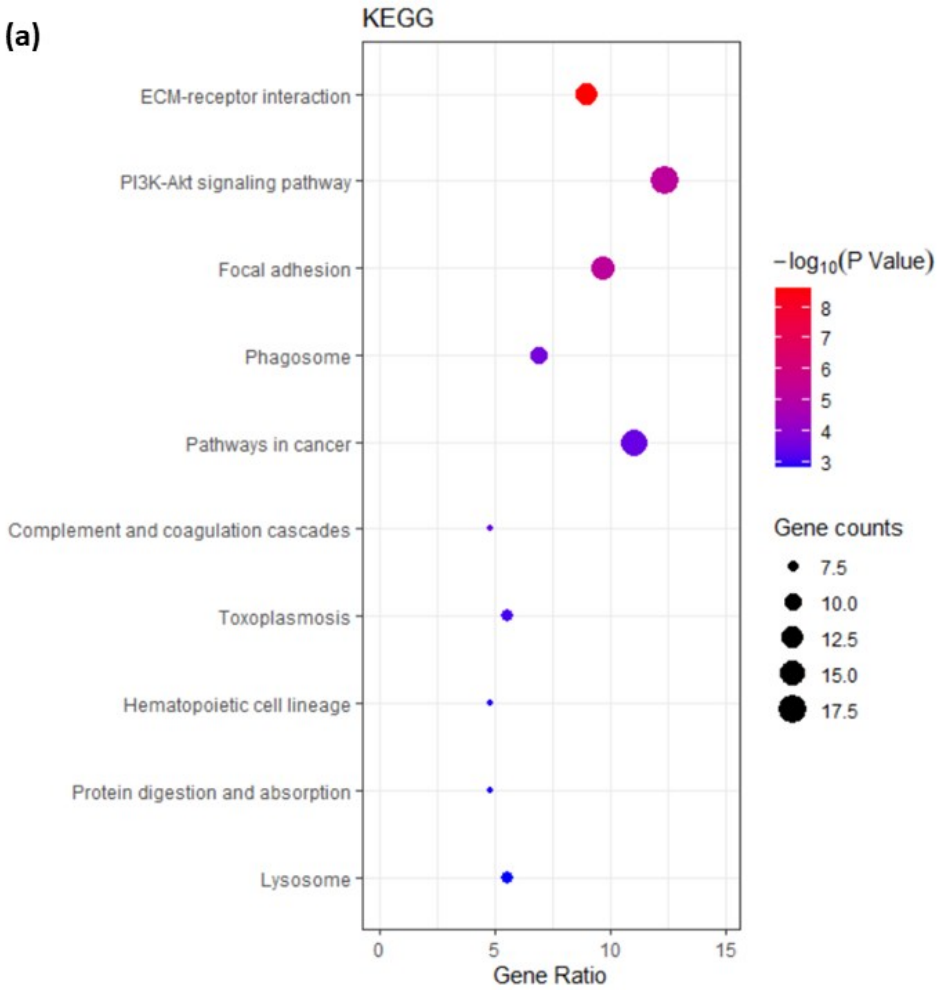
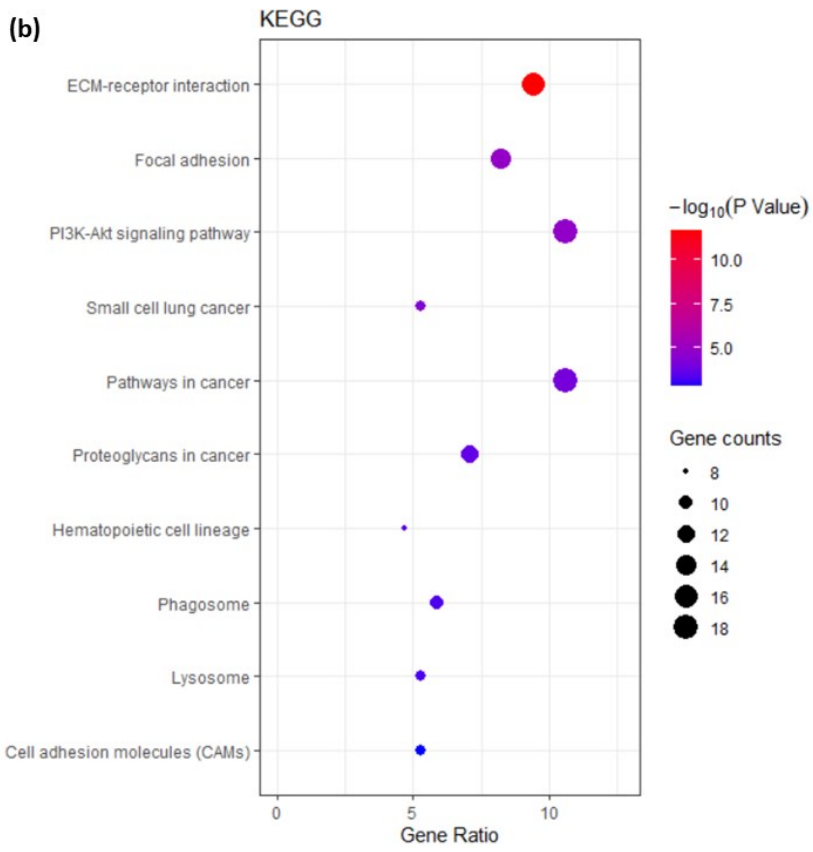


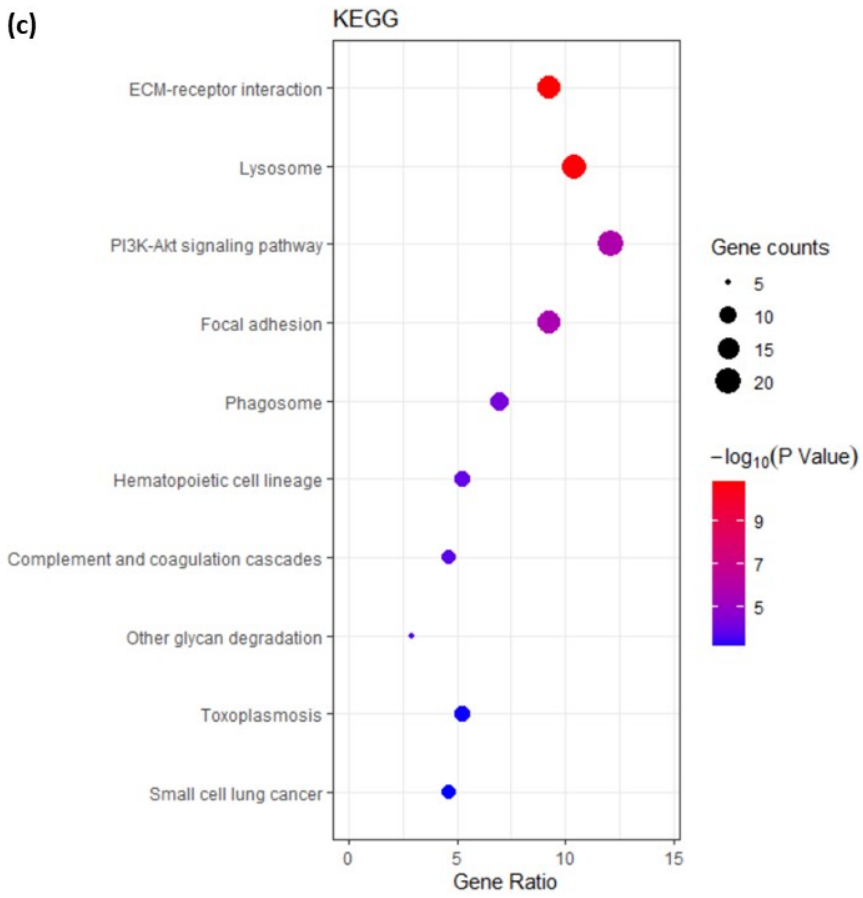
Figure S13. MALDI-TOF mass spectra of tryptic digested HRP after enrichment by first (a)/ third (b)/ fifth (c) recycled Fe_3O_4@TiO_2-GSH.


(a)
KEGG
ECM-receptor interaction
PI3K-Akt signaling pathway
Focal adhesion
Phagosome
Pathways in cancer
Complement and coagulation cascades
Toxoplasmosis
Hematopoietic cell lineage
Protein digestion and absorption
Lysosome
0
5
10
15
Gene Ratio
−log10(P Value)
8
7
6
5
4
3
Gene counts
7.5
10.0
12.5
15.0
17.5

144


(b)
KEGG
ECM-receptor interaction
Focal adhesion
PI3K-Akt signaling pathway
Small cell lung cancer
Pathways in cancer
Proteoglycans in cancer
Hematopoietic cell lineage
Phagosome
Lysosome
Cell adhesion molecules (CAMs)
0
5
10
Gene Ratio
−log10(P Value)
10.0
7.5
5.0
Gene counts
8
10
12
14
16
18

145



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147 **Figure S14**. The bubble chart about KEGG pathways of glycoproteins from Hela cells-

148 derived exosomes (a), MCF-7 cells-derived exosomes (b) and MDA-MB-231 cells-derived

149 exosomes(c). The closer the color is to red, the smaller the corresponding P value, and the

150 bigger the bubble, the more protein it contains.

151

Table S1. The detailed information of glycopeptides identified from HRP tryptic digest (100 fmol μL^{-1}) by Fe_3O_4@TiO_2-GSH. N# denotes the N-linked glycosylation site, * denotes pyroglutamylation on the N-terminal Q. GlcNAc=N-acetylglucosamine, Fuc=fuctose, Man=mannose, Xyl=xylose, Hex=hexose, HexNAc=N-acetylhexosamine.

Number	MH+ [Da]	Glycan composition	Amino acid sequence
H1	1842.6	XylMan3FucGlcNAc2	NVGLN#R
H2	2320.1	Man2GlcNAc2	MGN#ITPLTGTQGQIR
H3	2591.9	XylMan3FucGlcNAc2	PTLN#TTYLQTLR
H4	2611.9	XylMan3GlcNAc2	MGN#ITPLTGTQGQIR
H5	2851.1	FucGlcNAc	GLIQSDQELFSSPN#ATDTIPLVR
H6	3048.5	XylMan2GlcNAc2	SFAN#STQTFFNAFVEAMDR
H7	3089.1	XylMan3FucGlcNAc2	GLCPLNGN#LSALVDFDLR
H8	3208.0	XylMan3GlcNAc2	SFAN#STQTFFNAFVEAMDR
H9	3222.0	Man3FucGlcNAc2	SFAN#STQTFFNAFVEAMDR
H10	3323.1	XylMan3FucGlcNAc2	QLTPTFYDNSCPN#VSNIVR
H11	3355.1	XylMan3FucGlcNAc2	SFAN#STQTFFNAFVEAMDR
H12	3369.0	XylMan3FucGlcNAc2	SFAN#STQTFFNAFVEAM*DR
H13	3509.2	XylMan2FucGlcNAc2	GLIQSDQELFSSPN#ATDTIPLVR
H14	3526.3	XylMan3GlcNAc2	GLIQSDQELFSSPN#ATDTIPLVR

H15	3540.3	Man3FucGlcNAc2	GLIQSDQELFSSPN#ATDTIPLVR
H16	3606.3	XylMan3FucGlcNAc2	NQCRGLCPLNGN#LSALVDFDLR
H17	3672.3	XylMan3FucGlcNAc2	GLIQSDQELFSSPN#ATDTIPLVR
H18	3894.2	XylMan3FucGlcNAc2	LHFHDCFVNGCDASILLDN#TTSFR
H19	4056.1	XylMan3GlcNAc2	QLTPTFYDNSC(AAVESACPR)PN#VSNIVR-H2O
H20	4223.4	XylMan3FucGlcNAc2	QLTPTFYDNSC(AAVESACPR)PN#VSNIVR
H21	4851.7	Man3FucGlcNAc2, XylMan3FucGlcNAc2	LYN#FSNTGLPDPTLN#TTYLQTLR
H22	4984.8	XylMan3FucGlcNAc2, XylMan3FucGlcNAc2	LYN#FSNTGLPDPTLN#TTYLQTLR

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