Phosphoric acid functionalized mesoporous organo-silica (EPO) as the adsorbent for in-situ enrichment and isotope labeling of endogenous phosphopeptides

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Supporting Information

Experimental

Materials

1,2-bis(tri-methoxysilyl)ethane (BTME) and diethoxyphosphorylethyltriethoxysilane (PETES), $C_{18}H_{37}(OCH_2CH_2)_{10}OH$ (Brij-76), trypsin, β-casein (from bovine milk), 2,5-dihydroxybenzoic acid (DHB), formaldehyde (CH_2O, 37% vol/vol) and sodium cyanoborohydride ($NaBH_3CN$) were purchased from Sigma-Aldrich Company, Ltd. (USA); Formaldehyde (CD_2O, 20 wt%, 98% D), was purchased from Isotec (Miamisburg, OH); $Ti(SO_4)_2$ was obtained from Sinopharm (Shanghai, China); ammonia solution ($NH_3 \cdot H_2O$ 25%, vol/vol) was obtained from Shanghai Chemical Reagent; human serum from healthy volunteers and liver cancer patients were provided by Second Affiliated Hospital of Dalian Medical University using the standard clinical protocols and stored at −80 °C before the analysis; Acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water used for all experiments was purified with a Milli-Q water system (Millipore, Milford, MA). All other chemicals including hydrochloric acid (HCl), ethanol, sodium acetate and sodium chloride were of analytical grade.

Synthesis of Ti^{4+}-EPO

EPO was synthesized as previous reported,[13] Brij-76 (2.0 g) was dissolved in HCl

solution (32 ml, 2 M) and H_2O (16 ml) at 55 °C. Then a mixture of BTME and PETES was added to the solution, and the resulting mixture was stirred at 55 °C for 36 h. The solid product was recovered by filtration and dried at room temperature overnight. The as-synthesized material was refluxed in concentrated HCl (50 mL, 36.5 wt %) at 100 °C for 24 h to remove the template. The powder product was filtered and washed with deionized water to neutral and dried at 100 °C. The obtained sample was denoted as EPO-20, where 20 is the mol % of PETES/(PETES+BTME) in the initial gel mixture.

For loading metal ions of Ti^{4+}, 50 mg of EPO-20 was incubated in 25 ml of 100 mM $Ti(SO_4)_2$ solution with gentle stirring at room temperature overnight (18 h). The mixture was centrifugated and the supernatant was removed. Then the precipitate was washed with water and 200 mM NaCl to remove nonspecifically adsorbed Ti^{4+} and dried under vacuum at 30 °C for 12 h. The power was dispersed in 30% (vol/vol) ACN/ 0.1% TFA with a final concentration of 30 mg/ml.

Enrichment the tryptic digest of β-casein

β-casein was dissolved in ammonium bicarbonate buffer (100mM, pH 8.0) with a final concentration of 1 mg/ml (40 pmol/μL), and digested at 37 °C for 16 h with trypsin at the ratio of enzyme-to-substrate of 1: 40 (wt/wt). The capture of phosphopeptides by Ti^{4+}-EPO was described as follows. Firstly, 10 μL suspension of the Ti^{4+}-EPO was added into certain of the β-casein digest and 50 μL 50% ACN/2% TFA was added and incubated for 30 min. The supernatant was removed after centrifugation the suspension at 20, 000 g for 2 min. Then the resulting IMAC

material was respectively rinsed with 100 μL solutions containing 50% (vol/vol) ACN/0.1% TFA with 200 mM NaCl and 30% (vol/vol) ACN/ 0.1% TFA respectively.

Labeling of the phosphopeptides

In solution labeling The tryptic peptides were desalted, dried and dissolved in 25 μL sodium acetate buffer (100 mM, pH 5.7). And 2 μL of 4% (vol/vol) formaldehyde (CH_2O or CD_2O) was added and mixed softly, then 2 μL of 0.6 M sodium cyanoborohydride ($NaBH_3CN$) was added and shaken for 40 min. Quench the labeling reaction by adding 16 μL of 1% (vol/vol) ammonia solution, and 8 μL of formic acid was added to further quench the reaction and to acidify the sample. The mixtures were desalted and the labeled phosphopeptides were enriched using Ti^{4+}-EPO materials as described in the supporting information.

On column labeling The process was the same as previously reported.16 The tryptic peptides were loaded onto the C18 SPE column and desalted, and the peptides were labeled using 1 ml of the labeling reagents, and desalted again. Then the labeled phosphopeptides were enriched and detected.

In-situ labeling The phosphopeptides was enriched using Ti^{4+}-EPO beads to remove the non-phosphopeptides and salts. After dispersion the resulting IMAC materials into 25 μL sodium acetate buffer (100 mM, pH 5.7), 2 μL of 4% (vol/vol) formaldehyde (CH_2O or CD_2O) was added and mixed softly, then 2 μL of 0.6 M sodium cyanoborohydride ($NaBH_3CN$) was added and shaken for 40 min. The supernatant of the mixture was removed and the resulting material was washed with 25 μL of

deionized water, then the enriched phosphopeptides were eluted by 10 μL 10% (vol/vol) $NH_3 \cdot H_2O$ solution under sonication for 15 min. After an additional centrifugation (20, 000 g, 2 min), the supernatant was collected and analyzed by MALDI-TOF MS directly. The procedures for isolation and detection of phosphopeptides in human serum are shown in Scheme 1B, which was the same as tryptic digest of β-casein analysis excepting 1 μL serum was used.

References:

(13) P. Y. Wang, L. Zhao, R. Wu, H. Zhong, H. F. Zou, J. Yang and Q. H. Yang, *J. Phys. Chem. C*, 2009, **113**, 1359-1366.

Table S1. The signal to noise (S/N) of 2 of the phosphopeptides from β-casein digest labeled using in solution, on C18 column and in-situ strategy, respectively.

Peptide Sequence	Signal to Ratio (S/N)		
	In solution labeling	C18 column labeling	In-situ labeling
P1 *FQpSEEQQQTEDELQD*	19.5	11.4	54.9
P2 *FQpSEEQQQTEDELQD*KIHPF	18.8	16.5	41.2

Table S2. Endogenous phosphopeptides ions identified and quantified from human serum, and their observed ratio detected by MALDI-TOF-MS for 10 healthy people and 10 patients with liver cancer (HCC) serum. 1 μL of individual serum (HCC or normal) was labeled by light isotope regents as the sample and 1 μL of the mixture serum from 100 healthy people as the control was labeled with heavy isotope regents. Then the labeled sample were mixed and detected by MALDI-TOF-MS, and the ratios were obtained by the intensity of the peaks.

Peptides	Molecular Weight (Da)	Peptides sequences	Average ratio (L/H)	
			Normal (n=10)	HCC (n=10)
F1	1389.3	ADpSGEGDFLAEGGGV	1.08	0.43
F2	1460.4	DpSGEGDFLAEGGGV	1.17	0.52
F3	1545.2	DpSGEGDFLAEGGGVR	1.05	1.07
F4	1616.4	ADpSGEGDFLAEGGGVR	1.35	3.07

Figure S1. Pore size distribution of EPO materials calculated from the adsorption branch of the nitrogen adsorption/desorption isotherm. Inset: TEM image of the corresponding EPO materials.

Figure S2. Mass spectra of the labeling of phosphopeptides from β-casein (2 pmol for labeling and 100 fmol for the detection) with different time of 5min (A), 10min (B), 20min (C), 30min (D), 40min (E) and 60min (F). (β_1, FQpSEEQQQTEDELQDK; β_2, FQpSEEQQQTEDELQDKIHPF; β_3, RELEELNVPGEIVEpSLpSpSpSEESITR; β* indicates the labeled phosphopeptides; and $\beta^{\#}$, $\beta^{\#\#}$ stand for the incomplete labeling the N-terminus and ε-amino group of lysine of the phosphopeptides).

Figure S3. MALDI mass spectra of the labeled phosphopeptides from tryptic digest of β-casein (1 pmol/μL, 2 μL) for with the free solution isotope labeling (A and C light labeled), the on C18 column labeling (B and D light labeled), and the in-situ Ti^{4+}-EPO nanoparticles labeling (heavy labeled), respectively.

Figure S4. MALDI mass spectra of labeled phosphopeptides of β-casein enriched and deviated on Ti^{4+}-EPO beads: m/z 2117.5/2125.5 (*FQpSEEQQQTEDELQ D*K , stars indicated labeling sites). The samples were prepared using initial H: D mixing volume (1 pmol/μL) ratio of 1:1, 2:1, 4:1, and 10:1 , respectively (A); the resulting linearity plot for the peptide ion presented (B) also indicates these good correlations, with R^2 values as high as 0.9954.

Figure S5. Scatter plots demonstrating the ratio distribution of the four endogenous phosphopeptides in 10 serum samples of HCC and 10 of normal using 100 healthy adult mixtures as the control. (A) F1-F2 peptides identified in the sample serum; (B)

F3-F4 peptides identified in the sample serum (Detail sequences were shown in Table S2).

Figure S6. Partial least squares-discriminate analysis (PLS-DA) score plot deserved from the ratio of the sample (10 healthy people and 10 HCC patients) to the control (mixtures from 100 healthy people) obtained by MALDI-TOF-MS for endogenous phosphopeptides in serum, and the separation between the human liver caner and healthy groups (t[1] and t[2] were the new parameters deserved from the four phosphopeptides ratios).

Figure S1.

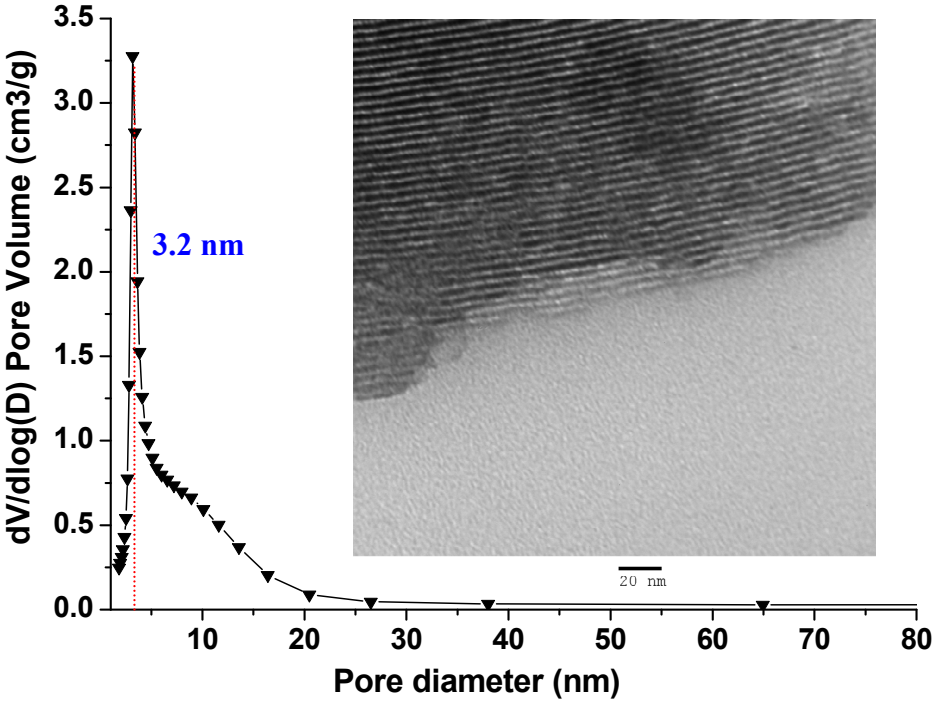
dV/dlog(D) Pore Volume (cm3/g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3.2 nm
20 nm
10
20
30
40
50
60
70
80
Pore diameter (nm)

Figure S2.

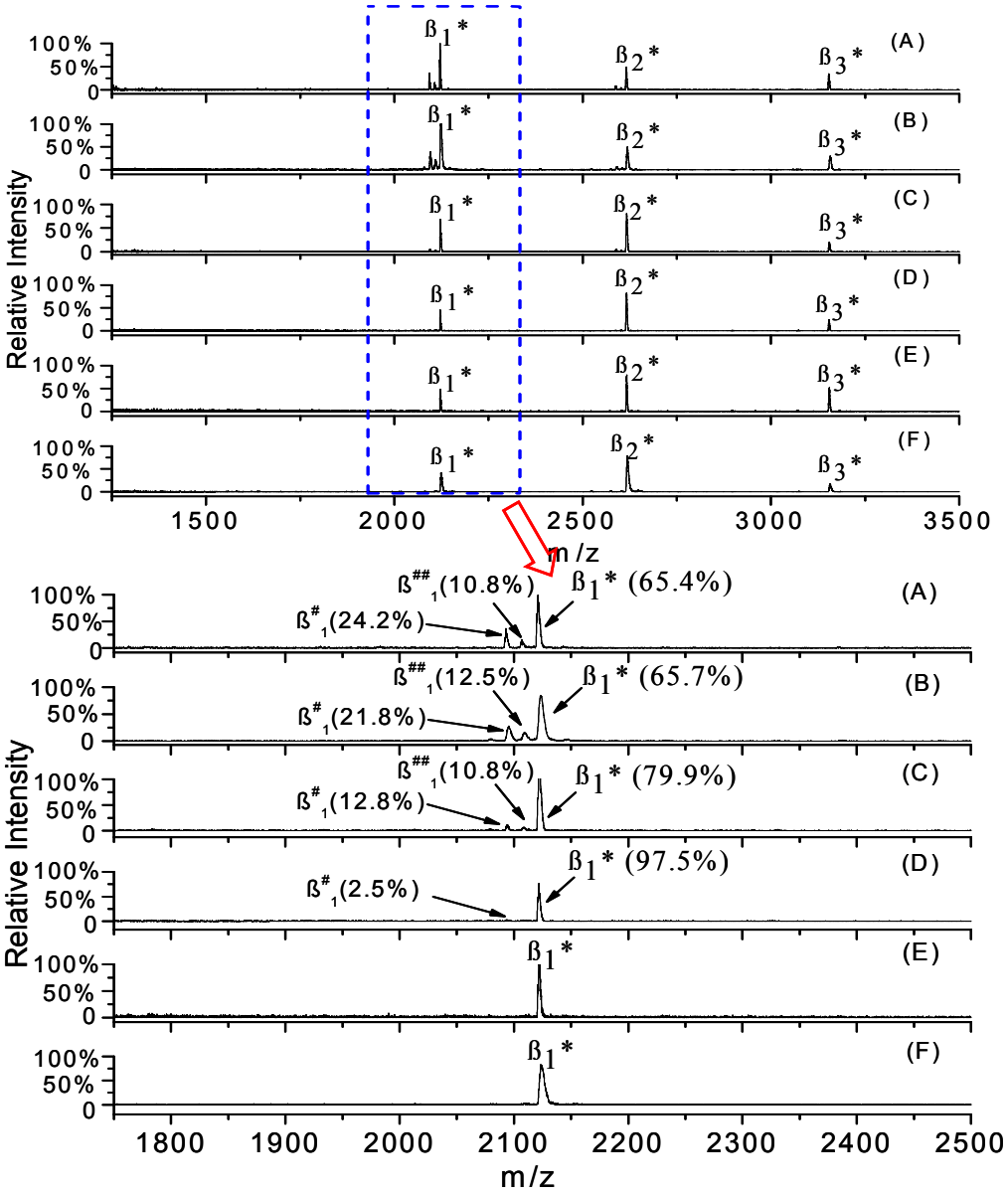
Relative Intensity
100%
50%
0
ß1*
ß2*
ß3*
(A)
(B)
(C)
(D)
(E)
(F)
1500
2000
2500
3000
3500
m/z
ß##1(10.8%)
ß1* (65.4%)
ß#1(24.2%)
ß##1(12.5%)
ß1* (65.7%)
ß#1(21.8%)
ß##1(10.8%)
ß1* (79.9%)
ß#1(12.8%)
ß1* (97.5%)
ß#1(2.5%)
1800
1900
2000
2100
2200
2300
2400
2500

Figure S3.

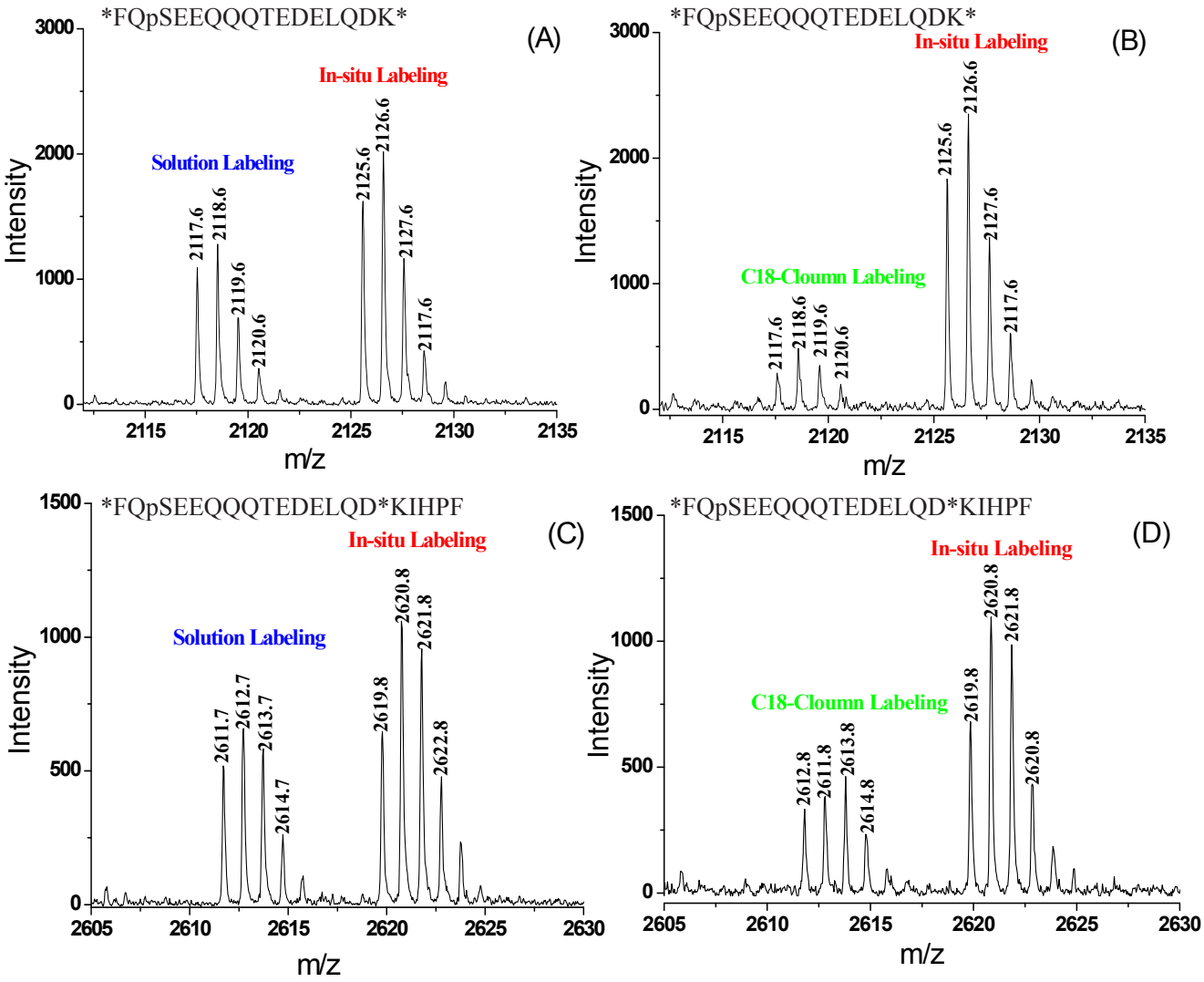
FQpSEEQQQTEDELQDK
(A)
In-situ Labeling
Solution Labeling
2117.6
2118.6
2119.6
2120.6
2125.6
2126.6
2127.6
2117.6
Intensity
m/z
FQpSEEQQQTEDELQDK
(B)
In-situ Labeling
C18-Cloumn Labeling
2117.6
2118.6
2119.6
2120.6
2125.6
2126.6
2127.6
2117.6
Intensity
m/z
*FQpSEEQQQTEDELQD*KIHPF
(C)
In-situ Labeling
Solution Labeling
2611.7
2612.7
2613.7
2614.7
2619.8
2620.8
2621.8
2622.8
Intensity
m/z
*FQpSEEQQQTEDELQD*KIHPF
(D)
In-situ Labeling
C18-Cloumn Labeling
2612.8
2611.8
2613.8
2614.8
2619.8
2620.8
2621.8
2620.8
Intensity
m/z

Figure S4.

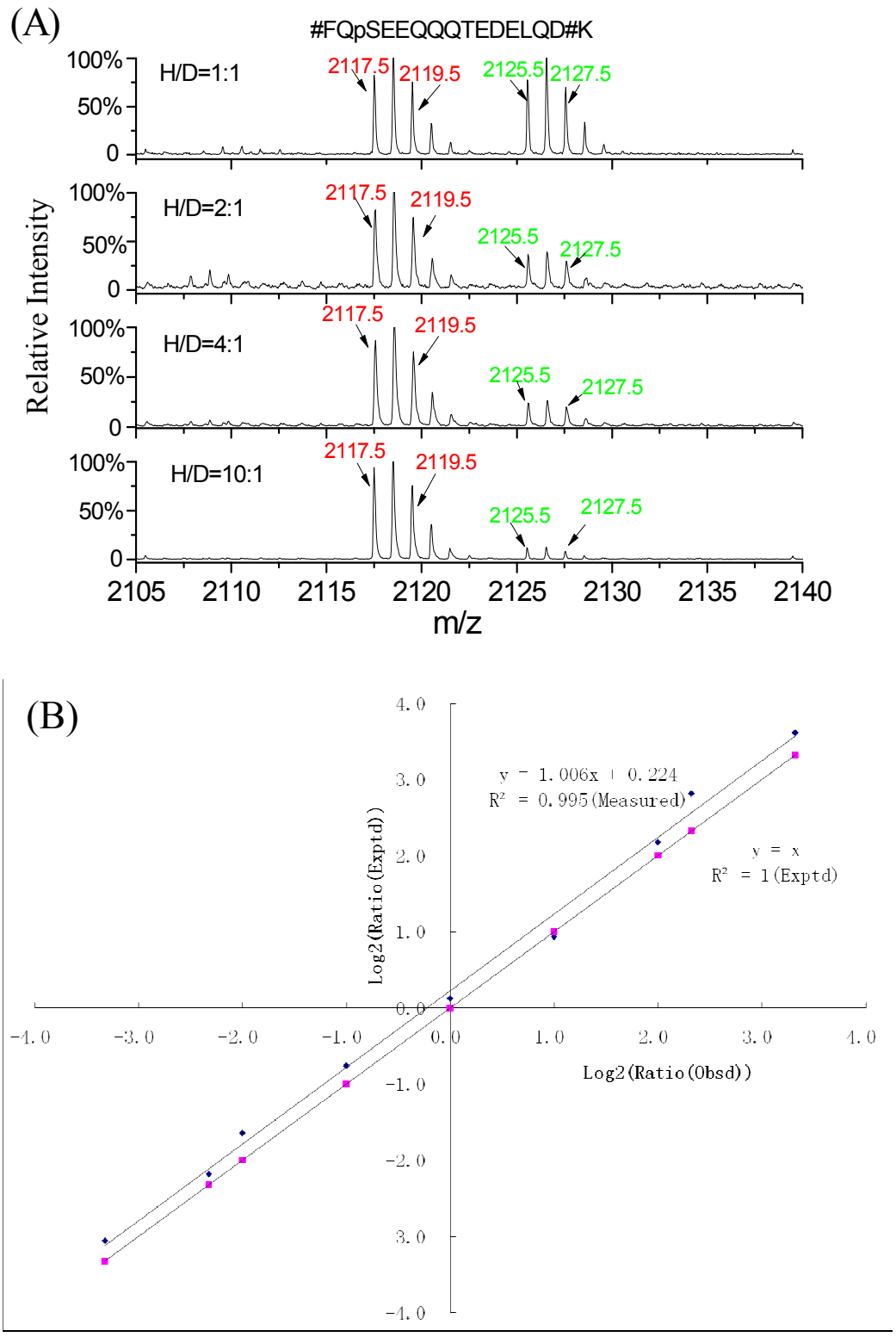
(A)
#FQpSEEQQQTEDELQD#K
H/D=1:1
H/D=2:1
H/D=4:1
H/D=10:1
2117.5
2119.5
2125.5
2127.5
100%
50%
0
Relative Intensity
2105
2110
2115
2120
2125
2130
2135
2140
m/z
(B)
y = 1.006x + 0.224
R² = 0.995(Measured)
y = x
R² = 1(Exptd)
Log2(Ratio(Exptd))
Log2(Ratio(Obsd))
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
3.0
-4.0
-4.0
-3.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0

Figure S5.

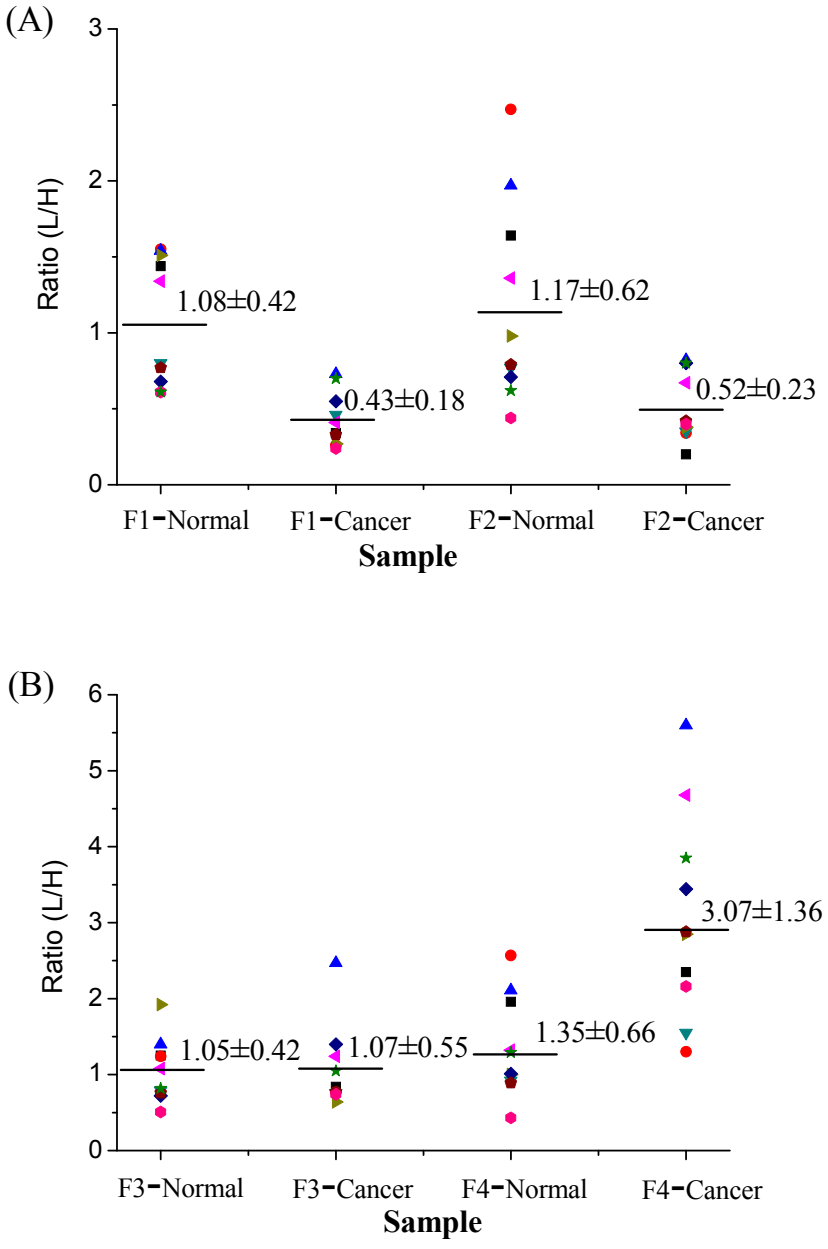

Figure S6.