

Table S1. 10 LC-MS injections to characterize Hpt site-specific glycosylation status

LC-MS Runs	Sample	Experiment	Purposes/Results
1-3	Hpt tryptic digest	Data dependent LC-CID/ETD-MS/MS	Glycopeptide precursors m/z(s) determination, ETD-MS/MS sequencing of peptides moiety (examples include T1-03 and T3-07), CID-MS/MS for glycan (T1-01, T1-05, T1-06, T1-07, T2 glycan composition, T3-01, T3-02, T3-03, T3-04, T3-05, T3-06, T3-08)
4-7	Hpt tryptic digest	Targeted LC-CID/ETD-MS/MS	Targeted CID-MS/MS for low abundance glycopeptides T1-02, T1-04, T3-07 and T3-09. Targeted ETD-MS/MS for abundant T2 glycopeptides T2-01 and T2-02. Targeted CID-MS/MS on fucosylated glycopeptides to confirm the position-specific fragments (both B and Y ions).
8-10	PNGase-F treated tryptic digest	Data dependent PLOT LC-CID-MS/MS	Site occupancy for T3

Table S2. Theoretical mass and m/z matrix for the identified site-specific glycoforms

Glycopeptide	Peptide mass	Glycoform [#]	Glycan Structure*	Glycoform mass	Glycoform m/z		
					z=3	z=4	z=5
T1	2681.1	T1-01	2-1S-0F	4595.8	1532.9	1149.9	920.2
		T1-02	2-1S-1F	4741.9	1581.6	1186.5	949.4
		T1-03	2-2S-0F	4887.0	1630.0	1222.8	978.4
		T1-04	2-2S-1F	5033.2	1678.7	1259.3	1007.6
		T1-05	3-2S-0F	5252.4	1751.8	1314.1	1051.5
		T1-06	3-3S-0F	5543.6	1848.9	1386.9	1109.7
		T1-07	3-3S-1F	5689.8	1897.6	1423.4	1139.0
T2	1459.6	T2-01	(2-2S-0F)+(2-1S-0F)	5580.3	1861.1	1396.1	1117.1
		T2-02	(2-2S-0F)+(2-2S-0F)	5871.6	1958.2	1468.9	1175.3
		T2-03	(2-2S-0F)+(3-2S-0F)	6236.9	2080.0	1560.2	1248.4
		T2-04	(2-2S-0F)+(3-2S-1F)	6383.0	2128.7	1596.8	1277.6
		T2-05	(2-2S-0F)+(3-3S-0F)	6528.1	2177.0	1633.0	1306.6
		T2-06	(2-2S-0F)+(3-3S-1F)	6674.3	2225.8	1669.6	1335.9
		T2-07	(2-2S-0F)+(4-3S-0F)	6893.5	2298.8	1724.4	1379.7
		T2-08	(2-2S-0F)+(4-3S-1F)	7039.6	2347.5	1760.9	1408.9
		T2-09	(2-2S-0F)+(4-4S-0F)	7184.7	2395.9	1797.2	1437.9
		T2-10	(2-2S-0F)+(4-4S-1F)	7330.9	2444.6	1833.7	1467.2
T3	1796.1	T3-01	2-1S-0F	3710.8	1237.9	928.7	743.2
		T3-02	2-2S-0F	4002.1	1335.0	1001.5	801.4
		T3-03	2-2S-1F	4148.2	1383.7	1038.1	830.6
		T3-04	3-2S-0F	4367.4	1456.8	1092.9	874.5
		T3-05	3-2S-1F	4513.6	1505.5	1129.4	903.7
		T3-06	3-3S-0F	4658.7	1553.9	1165.7	932.7
		T3-07	3-3S-1F	4804.8	1602.6	1202.2	962.0
		T3-08	4-2S-0F	4732.7	1578.6	1184.2	947.5
		T3-09	4-3S-0F	5024.0	1675.7	1257.0	1005.8

[#], Glycoforms identified on Hpt. The structures of corresponding glycoforms can be found in Table S3.

^{*}, Glycan structure (text format) of corresponding glycoform. The first arabic number represents branching; second arabic number represents sialic acid number, "S" means sialic acid; the third arabic number represents fucose number, "F" means fucose.

Table S3. Site-specific glycan structures identified on Hpt*

T1-01	T1-02	T1-03	T1-04	T1-05	T1-06	T1-07
T2-01	T2-02	T2-03	T2-04	T2-05	T2-06	T2-07
T2-08	T2-09	T2-10	T3-01	T3-02	T3-03	T3-04
T3-05	T3-06	T3-07	T3-08	T3-09		

* key to symbols can be found in Figure 3.

Supplementary Material

Figure Captions

Figure S1. PLOT LC-LTQ-CID/ETD-MS system. (A) Scanning electron micrograph (SEM) of a monolith SPE column; (B) SEM of PLOT column; (C) Schematic representation of electrospray using the 10 μm i.d. PLOT column. (D) PicoClear tee allowed quick sample loading while minimizing band broadening. The 10-port valve was first set in the loading position, with tee **a** serving as splitter and tee **b** as connector. Sample was loaded onto the SPE column while salts and other ESI deleterious components were directed to waste. After loading, both the sample injection valve and 10-port valve were switched to the separation position (dashed line, Figure S1), and the SPE column was now positioned to be in-line with the PLOT separation column. The tees were reversed with tee **a** now serving as connector, while tee **b** acted as splitter.

Figure S2. Glycoform identification on glycopeptide T3. (A) ETD-MS/MS generated peptide fragments c and z' ions. (B) CID-MS/MS generated oxonium ions and glycopeptide fragments. (C) Averaged base peak chromatogram contained all the glycans on glycopeptide T3. m/z 1527.2 is a co-eluting nonglycosylated peptide. NL: normalized level. Key to symbols can be found in Figure 3.

Supplementary Material

Figure Captions

Figure S3. Glycan compositions identified on glycopeptide T2. The empty circle represents charge state +5, while the filled circle represents charge state +4. NL: normalized level. Key to symbols can be found in Figure 3.

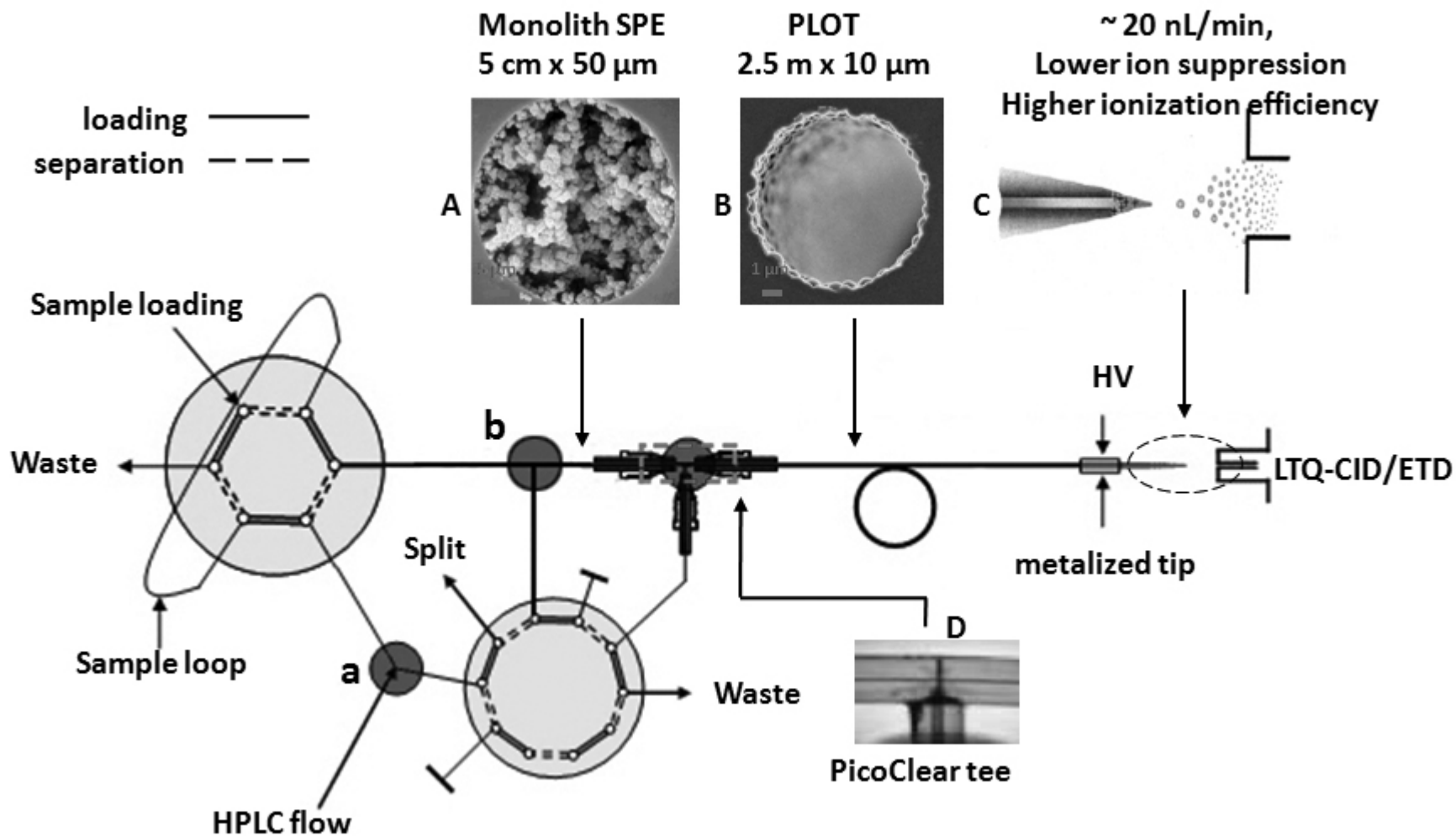


Figure S1

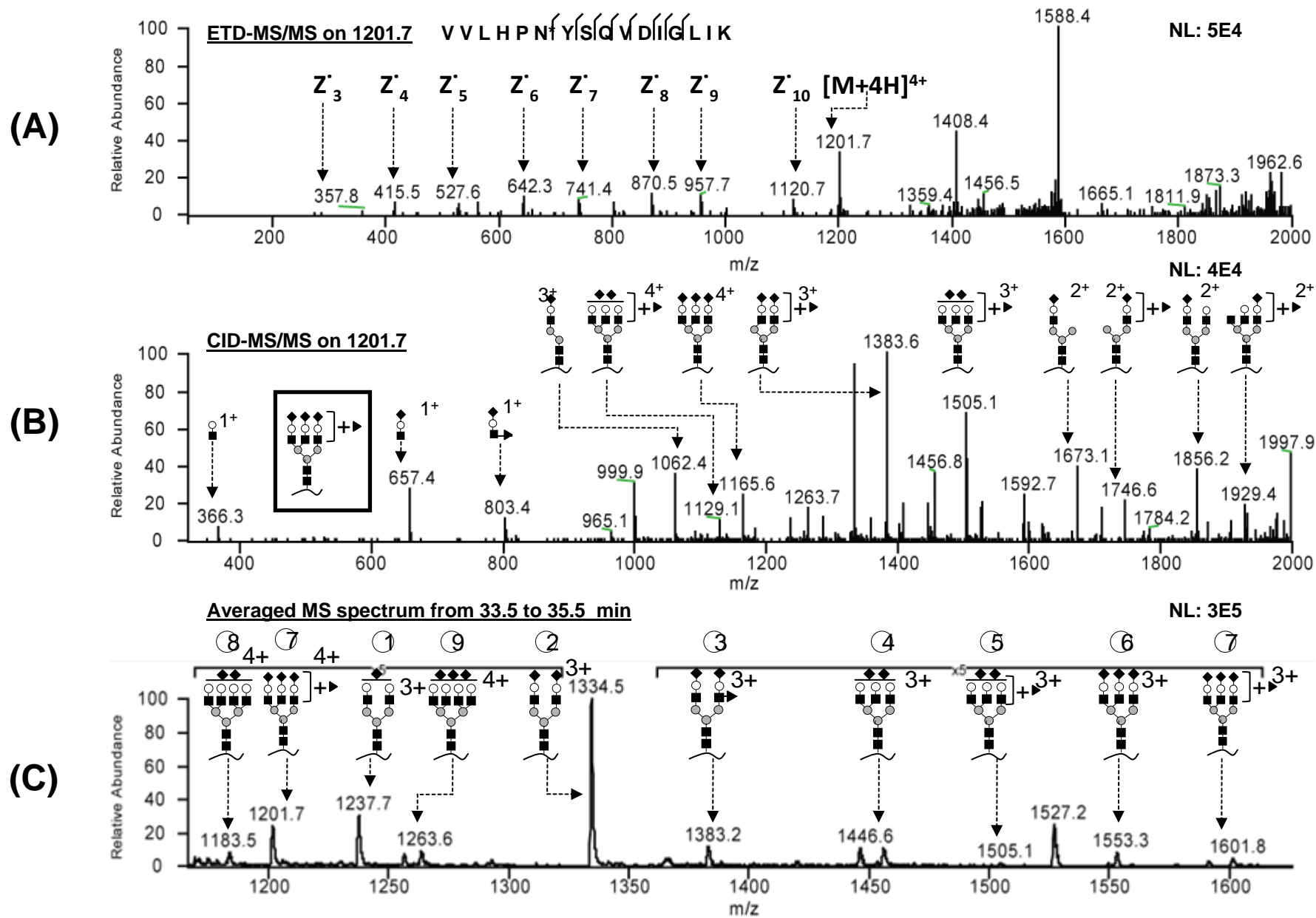


Figure S2

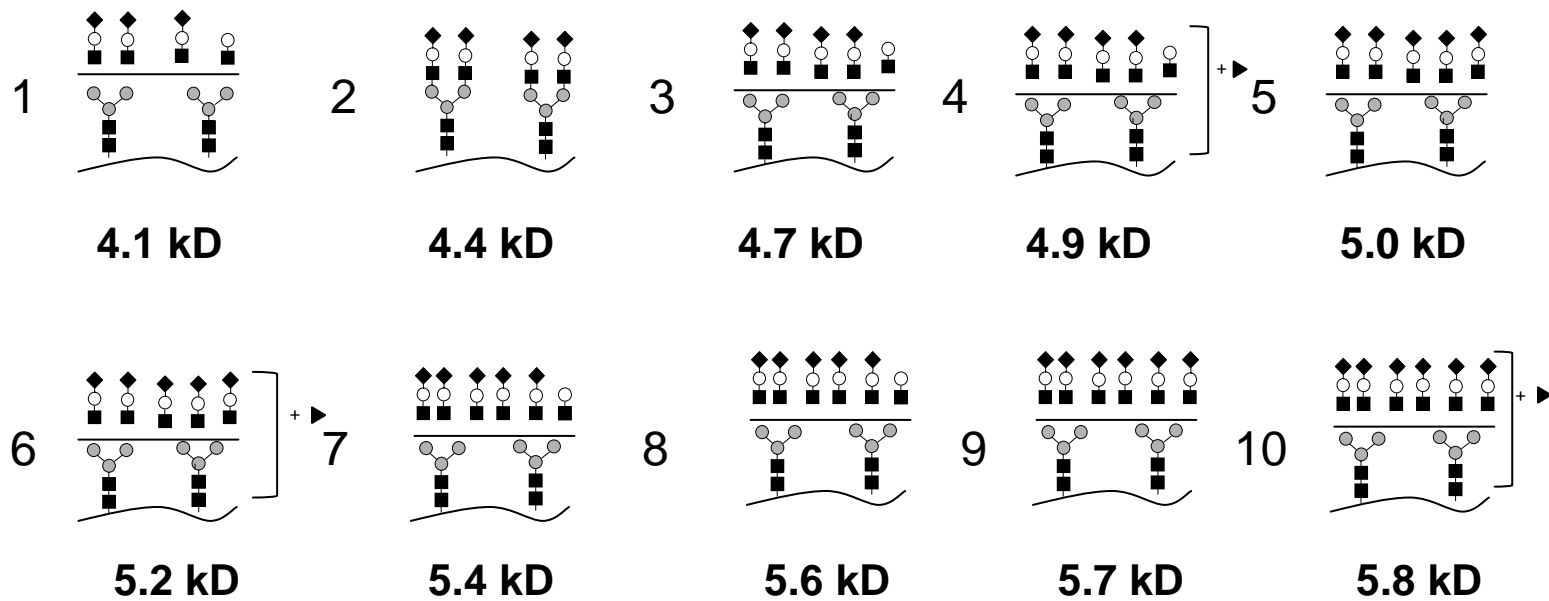
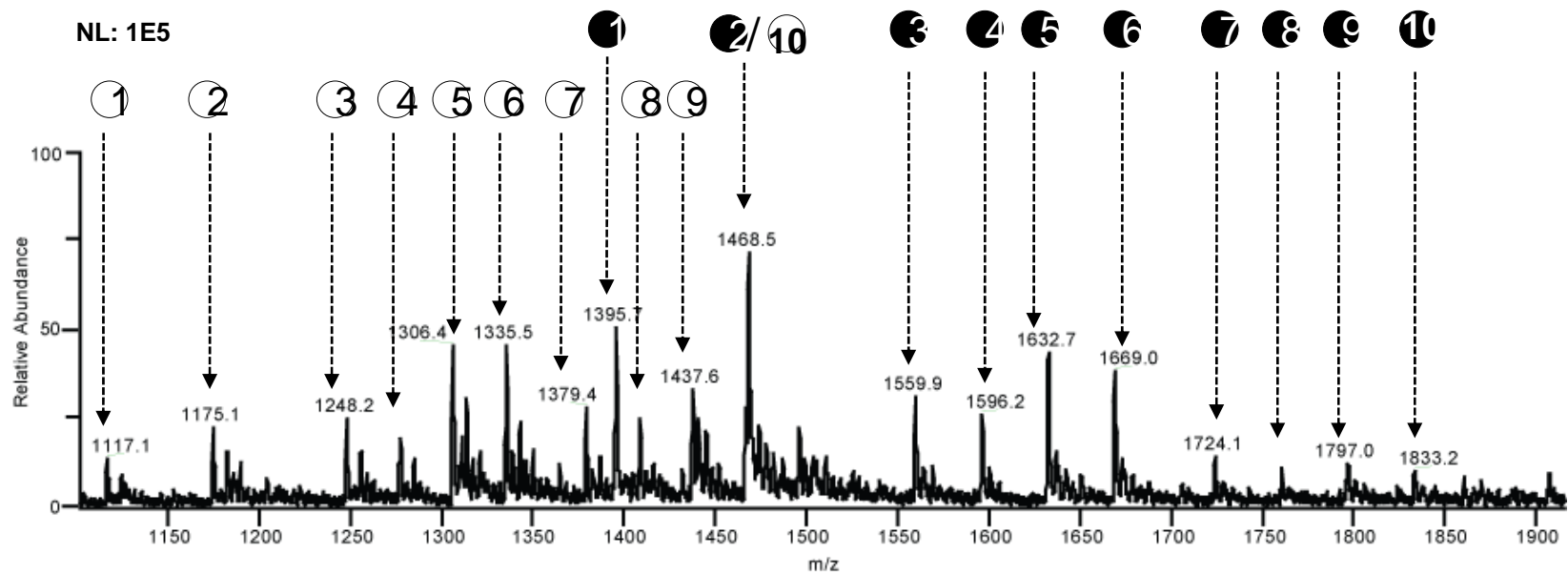


Figure S3