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Isotope-Coded Carbamidomethylation for Quantification of N-Glycoproteins with Online Microbore Hollow Fiber Enzyme Reactor-Nanoflow Liquid Chromatography-Tandem Mass Spectrometry

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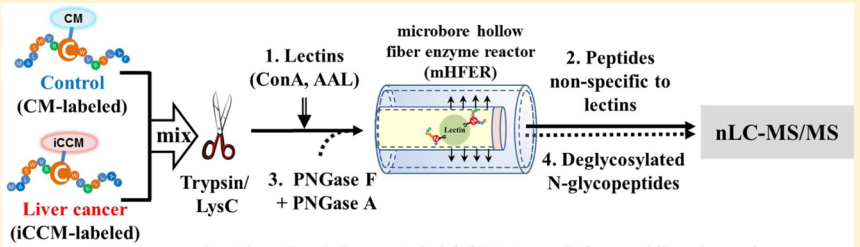
Supporting Information



ABSTRACT: This paper introduces a simple, inexpensive, and robust quantitative proteomic method for quantifying N-linked glycoproteins based on isotope-coded carbamidomethylation (iCCM) incorporated into an online microbore hollow fiber enzyme reactor and nanoflow liquid chromatography-tandem mass spectrometry (mHFER-nLC-MS/MS). The iCCM quantitation uses carbamidomethylation (CM; a routine protection of thiol groups before proteolysis) of the Cys residue of proteins with iodoacetamide (IAA) or its isotope (IAA-$^{13}C_2$,D_2: 4 Da difference). CM-/iCCM-labeled proteome samples are mixed for proteolysis; then, online enrichment of N-glycopeptides using lectin affinity is carried out in an mHFER before nLC-MS/MS for quantification using multiple reaction monitoring (MRM). Initial evaluation of the iCCM method varying the mixing ratio of CM-/iCCM-labeled bovine serum albumin (BSA) standards yielded successful quantification of 18 peptides with less than 2% variation in the calculated ratio of light/heavy-labeled peptides. The iCCM quantitation with mHFER-nLC-MS/MS was evaluated with three standard glycoproteins (α-1-acid glycoproteins, fetuin and transferrin) and then applied to serum glycoproteins from liver cancer patients and controls, resulting in successful quantification of 73 N-glycopeptides (from 49 N-glycoproteins), among which 19 N-glycopeptides from 14 N-glycoproteins showed more than a 2.5-fold aberrant change in liver cancer patients' sera compared with the pooled control. Although iCCM quantitation with mHFER-nLC-MS/MS applies only to glycopeptides with Cys residue, the method can offer several advantages over other labeling methods when applied to targeted glycoproteins: The iCCM method does not require an additional labeling reaction under special conditions nor complicated procedures to purify labeled products using additional columns. Isotope labeling at the protein level can minimize potential uncertainty originating from unequal efficiencies in protein digestion in separate vials and retrieval of each labeled peptide when labeling takes place at the peptide level. In addition, the labeling reagents for the iCCM method are readily obtained at a reasonable cost, which can make protein quantification easily accessible.

Mass spectrometry (MS)-based proteomics has become a fundamental strategy in identifying proteins and studying their diverse functions in complicated biological systems such as cells, sera, tissues, and microorganisms.[1−3] MS alone or tandem MS-based analyses together with on-/off-line liquid chromatography (LC) are useful tools for the quantitative determination of targeted protein(s) of interest, and protein quantification can be used to understand metabolic functions. In particular, N-linked glycosylation, a common post-translational modification of proteins through the covalent attachment of a microheterogeneous monosaccharide to asparagine (Asn or N) with the consensus motif of N-X-S/T (X for any amino acid except proline), affects protein structure and function in ways that induce changes in cell signaling, cell differentiation, and

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proliferation.[4,5] Quantitative profiling of N-glycoproteins from human sera or tissues with clinical origins has provided promising results in which N-glycosylation can be a key determinant concerning either dysfunction in a cell or virulence factors related to human diseases or cancers.[6−9]

Although sophisticated MS techniques have facilitated the study of N-glycoproteins, the complexity of glycosylation patterns and rarity of glycoproteins still present challenges. Strategies to enhance isolation or enrichment of N-linked glycoproteins/glycopeptides from complicated mixtures have been developed using hydrazide chemistry,[10,11] titanium dioxide beads,[12] and hydrophilic interaction liquid chromatography (HILIC).[4,13] The selective affinity of lectin for carbohydrates (i.e., mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, and fucose) is commonly used with diverse platforms such as cartridge, membrane, column, and flow field-flow fractionation to enrich glycosylated species.[14−18] Recently, we developed a microbore hollow fiber enzyme reactor (mHFER)[19] for online proteolysis using a very small (∼10 μL) hollow fiber membrane with enhanced proteolytic efficiency before nanoflow LC-MS/MS analysis. Online microbore hollow fiber enzyme reactor and nanoflow liquid chromatography-tandem mass spectrometry (mHFER-nLC-MS/MS) can selectively enrich N-linked glycopeptides using lectin affinity. Briefly, a mixture of tryptic peptides and lectin are loaded to the mHFER in a continuous flow. Peptides with no affinity to lectin pass through the porous HF membrane wall toward nLC-MS/MS for a breakthrough characterization of peptides (or waste) while glycopeptide-lectin complexes remain within it. Then, an injection of endoglycosidase (i.e., PNGase F/A) into the HF reactor induces deglycosylation of glycopeptides from lectin, resulting in the elution of deglycosylated peptides to a trapping column for online nLC-MS/MS analysis. While mHFER-nLC-MS/MS exhibits potential for the study of glycoprotein biomarkers in an online operation, a suitable quantification method is required to handle lectin-specific N-glycopeptides.

Several stable isotope-labeling methods have been successfully applied to N-glycoproteins for quantitative analysis: an isobaric tag for relative and absolute quantification (iTRAQ),[20,21] a mass differential tag for relative and absolute quantification (mTRAQ),[22,23] tandem mass tags (TMT),[24,25] and reductive dimethylation.[26,27] Although these isotope-labeling strategies provide robust quantification of proteins with high precision and accuracy, they require additional buffer exchange steps to deplete reagents, such as ammonium bicarbonate, ammonium citrate, and tris buffers, that affect the yield by incorporating heavy-/light-coded tracer into the targeted protein or peptide site(s). Moreover, because most isotope tagging methods are carried out at the peptide level, they can elicit quantitative variations of proteolytic peptides due to unequal vial-to-vial enzyme activity or unequal peptide retrieval as well as nonspecific isotope tagging of peptide side chain amines, including hydroxyl residues such as serine and tyrosine.

This study introduces a simple inexpensive isotope labeling method, isotope-coded carbamidomethylation (iCCM), that can be used with mHFER-nLC-MS/MS[19] for online enrichment of N-glycopeptides followed by reproducible quantification of lectin-specific N-glycopeptides. iCCM is based on carbamidomethylation (CM) of cysteine residue at the protein level, which is commonly carried before proteolysis to protect thiol groups. It uses iodoacetamide (IAA) and its isotope (IAA-$^{13}C_2$, D_2) for light-/heavy-labeling of protein samples, inducing a mass difference of +4 Da per each cysteinyl residue. Therefore, proteolysis is carried out with a mixture of light-/heavy-labeled protein samples as illustrated in Figure S1 of the Supporting Information, which can reduce uncertainty in the retrieval of digested peptides compared to separate digestion followed by isotope labeling at the peptide level. For the quantitative analysis of N-glycopeptides using mHFER-nLC-MS/MS, the digested mixture of labeled peptides is mixed with lectin for lectin-glycopeptide complexation and loaded into the mHFER as described above. Therefore, deglycosylated CM/-iCCM-labeled peptides can be quantitatively analyzed by nLC-MS/MS using multiple reaction monitoring (MRM).

The iCCM method's capability for relative quantification was tested first by varying the mixing ratio of CM- and iCCM-labeled bovine serum albumin (BSA), followed by digestion and nLC-MS/MS analysis. Then, iCCM-based quantification of glycopeptides with mHFER-nLC-MS/MS was evaluated with α-1-acid glycoprotein (AGP), a glycoprotein standard, by mixing CM- and iCCM-labeled AGP samples at different mixing ratios followed by digestion for subsequent online enrichment and analysis of deglycosylated CM/-iCCM-labeled peptides using concanavalin A (ConA). The developed method was applied to liver cancer and control sera using two lectins (ConA and *Aleuria aurantia* lectin (AAL)) for lectin-specific enrichment and iCCM-based quantification of targeted N-linked glycopeptides using mHFER-nLC-MS/MS.

EXPERIMENTAL SECTION

Materials and Chemicals. Iodoacetamide (IAA, C_2H_4INO), heavy iodoacetamide (IAA*, $^{13}C_2H_2D_2INO$), L-cysteine, tris buffer, sodium hydrogen phosphate, dithiothreitol (DTT), urea, calcium chloride, formic acid, ConA, N-glycosidase A (PNGase A), and protein standards (BSA and fetuin from bovine, AGP and transferrin from human plasma) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade trypsin, Lys-C, and N-glycosidase F (PNGase F) from *Elizabethkingia miricola* were purchased from Promega Corp. (Madison, WI, USA). For the selective affinity of core-fucosylated glycopeptides, AAL from Vector Laboratories, Inc. (Burlingame, CA, USA) was used. Capillary analytical columns and trapping columns were prepared with fused-silica capillaries of different inner diameters (i.d.) (20, 75, and 100 μm i.d., 365 μm o.d.) from Polymicro Technology (Phoenix, AZ, USA) using Magic C18AQ beads (3 μm, 100 Å and 3 μm, 200 Å) from Michrom Bioresources, Inc. (Auburn, CA, USA) as packing materials. All fittings, adapters, and PEEK tubes were purchased from Upchurch Scientific (Oak Harbor, WA, USA) of IDEX Health & Science. HPLC-grade water and acetonitrile from J. T. Baker (Deventer, Netherlands) were used to prepare nLC mobile phase solutions.

Human serum samples were obtained from 10 healthy adults (control) and three patients with hepatocellular carcinoma after a 10 h fast under written consent from Severance Medical Center (Seoul, Korea). Control samples were pooled together, but patient samples were analyzed individually.

iCCM Labeling and Digestion Procedure. For protein denaturation, 20 μg of each protein standard (BSA and AGP) or human plasma sample dissolved in 0.1 M phosphate-buffered saline (PBS) solution containing 8 M urea and 10 mM DTT was incubated for 2 h at 37 °C. Two aliquots (10 μg) of each sample were prepared for an efficiency test of the iCCM labeling. Thiol groups at Cys residue were protected by CM

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using 20 mM IAA for one aliquot and 20 mM IAA* ($^{13}C_2H_2D_2INO$) for the other aliquot, both in an ice bath for 2 h in the dark. Excess IAA and IAA* were removed by adding cysteine (40-fold access of IAA); each mixture was vortexed for 30 min and then diluted to a final concentration of 1 M urea in 0.1 M PBS. Both CM- and iCCM-labeled protein standard solutions were mixed together, varying the mixing ratio. Then, each mixture was digested in series using sequencing-grade trypsin and Lys-C (1/40 of target protein amount) and incubated for 24 h at 37 °C. The resulting peptides were retrieved from the mixture by desalting the solution with an Oasis HLB cartridge from Waters (Milford, MA, USA) and lyophilized for storage in the freezer.

mHFER-nLC-MS/MS. The structure of the mHFER is shown in Figure 1; its assembly is the same as reported in a previous study.[19] Details are in the Supporting Information.

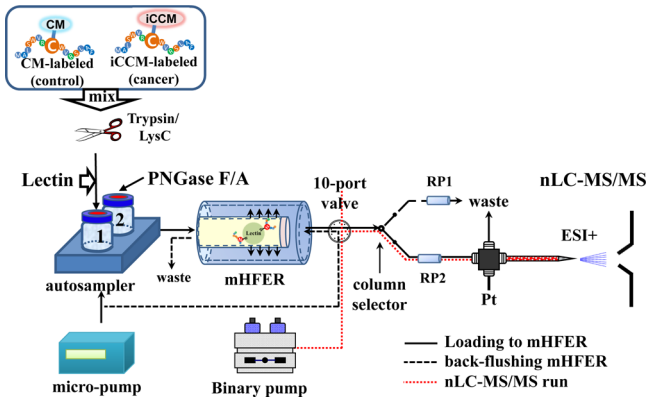

Figure 1. iCCM with mHFER-nLC-MS/MS analysis for online enrichment of lectin-bound N-glycopeptides and relative quantitation. (1) Injection of mixtures of CM-/iCCM-labeled peptide digests and lectins into the mHFER module and depletion of peptides unbound to lectins from mHFER to be trapped in RP1; (2) loading of PNGase F (or A) from the autosampler into mHFER for deglycosylation of N-glycopeptides from lectins to be trapped in an RP2 trap column (solid line). During nLC-MS/MS analysis (dotted line), mHFER is washed off by a back-flushed flow (broken line).

Enrichment and Quantification of Labeled N-Glycopeptides by mHFER-nLC-MS/MS. For online enrichment of N-glycopeptides, a mixture of digested CM-/iCCM-labeled peptide samples was mixed with lectins for lectin-specific binding of N-glycopeptides, and the whole mixture was loaded into the mHFER at 5 μL/min using an external micropump, Ultimate 3000 HPLC pump from Thermo Scientific Dionex (Waltham, MA, USA), connected to the autosampler (see the solid line configuration in Figure 1). A mixture of CM-/iCCM-labeled AGP peptide digests was mixed with ConA at a weight ratio of 1:20 (target protein/lectin) in a binding buffer (50 mM tris buffered saline (TBS) at pH 7.40 containing 1 mM calcium chloride, 1 mM manganese chloride, and 1 mM magnesium chloride) and left for 30 min at room temperature to form ConA-glycopeptide complexes. When the mixture was loaded to the mHFER, peptides unbound to lectins passed through the pores of the mHF membrane and were discarded or accumulated in the RP1 trap for nLC-MS/MS analysis. Deglycosylation of N-glycopeptides from the ConA-glycopeptide complex was made by introducing 5 μL of PNGase F (≥5000 units/mL) into the mHF from the autosampler. The deglycosylated peptides that eluted from the wall of the mHF membrane were transferred to the RP2 trap (see dotted line configuration in Figure 1). Then, nLC-MS/MS analysis of the mixture of labeled deglycosylated peptides could be made in sequence to determine the relative ratio of CM-/iCCM-labeled peptides using the MRM method. While the LC-MS/MS run was accomplished, the remaining enzymes and protein residues in the mHFER were back-flushed by flow from the micropump (broken line connection in Figure 1) for at least 30 min.

The same procedure was applied to the human blood serum samples from a pooled control (CM-labeled) and three hepatocellular carcinoma sera (iCCM-labeled). Each plasma sample was treated with ProteoPrep Immunoaffinity Albumin & IgG Depletion Kit from Sigma-Aldrich to deplete albumin and IgG prior to labeling. The concentration of eluted plasma proteins after depletion was measured by the Bradford assay using Bradford reagent from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Proteolysis of the labeled protein mixtures followed the same process described above. For the selective isolation of serum N-glycopeptides, two types of lectin (AAL for core-fucosylated glycopeptides and ConA for mannose) were used separately, and two types of N-glycosidase (PNGase F and PNGase A) were used in sequence to ensure deglycosylation of N-glycopeptides from the lectin. The procedures for the subsequent analysis of the whole mixture (lectin + CM-/iCCM-labeled peptides) with mHFER-nLC-MS/MS were the same as those described for the efficiency test using AGP above.

RESULTS AND DISCUSSION

Evaluation of the iCCM Quantitation Method for Relative Quantitative Proteomics. The iCCM method for quantitative proteomic analysis was first evaluated using BSA to check its precision in incorporating the light and heavy IAA into the target cysteinyl residue and to compare the experimental ratio of CM-/iCCM-labeled peptides with the expected ratio. Tests with BSA were made by varying the mixing ratio of CM-labeled and iCCM-labeled BSA solutions (10:0, 8:2, 5:5, 2:8, and 0:10 in volume ratio) followed by in-solution digestion of each mixture and subsequent nLC-MS/MS analysis to calculate the peak area of labeled peptides for comparison. A total of 41 peptides were identified by nLC-MS/MS analysis. Table S1, Supporting Information, lists 18 peptides having at least one cysteinyl residue that were assigned CM-/iCCM-labeled peptide pairs with mass differences of 4, 8, and 12 Da for singly, doubly, and triply labeled peptides, respectively. The calculated ratio of each peptide pair was obtained from the extracted peak area of the precursor ions. It was found that cysteinyl residues were fully converted to CM-/iCCM-labeled forms through carbamidomethylation without detecting any mislabeled peptide. The average value of the calculated ratio at three mixing conditions was 4.02 ± 0.09 (4:1), 1.02 ± 0.03 (1:1), and 0.25 ± 0.01 (1:4), showing less than 2% variation. Among the 18 Cys-containing peptides in Table S1, Supporting Information, the four peptides at the bottom had more than two cysteine residues representing the exact difference in observed m/z values depending on charge states; however, the calculated ratio of each peptide pair showed similar values compared to those of other peptides with a single cysteine. For instance, Figure 2a contains five MS spectra showing the comparison of CM-labeled (YNGVFQEccQAEDK, m/z 874.36, $[M + 2H]^{2+}$) and iCCM-labeled peptides (YNGVFQEc*c*QAED, m/z 878.37, $[M + 8 + 2H]^{2+}$) obtained at five different mixing conditions. Extracted

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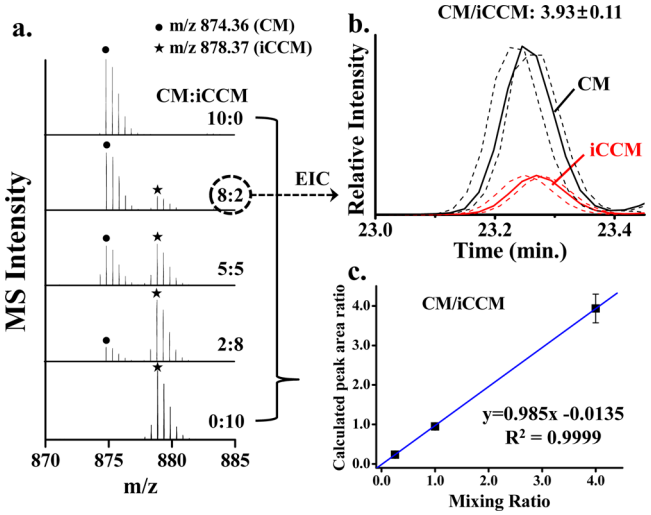

Figure 2. nLC-MS/MS analysis of CM-/iCCM-labeled BSA peptides. (a) MS spectra showing the comparison of MS peaks between CM-labeled (YNGVFQE**cc**QAEDK, m/z 874.36, +2, t_r = 23.24 ± 0.02 min) and iCCM-labeled (YNGVFQE**c***Q**c***QAEDK, m/z 878.37, +2, t_r = 23.25 ± 0.02 min) peptides at five different mixing ratios, (b) superimposed extracted ion chromatograms of CM-/iCCM-labeled peptides showing an area ratio of 3.93 ± 0.11 for the 8:2 mixture, and (c) the linear relationship of calculated area ratios vs mixed ratios (CM/iCCM).

ion chromatograms (EICs) of each primary ion (marked with filled circles and stars) at an 8:2 mixing ratio were superimposed in Figure 2b, and the comparison of each peptide pair shows a slight variation in retention times (23.24 ± 0.02 min and 23.25 ± 0.02 min for CM-labeled and iCCM-labeled peptides, respectively, n = 3 for both) with an average peak area ratio of 3.93 ± 0.11 (or 7.97:2.03), which was very close to the mixing ratio of 4. Calculated peak area ratios for two other mixing conditions (5:5 and 2:8) were 1.01 ± 0.03 and 0.25 ± 0.01. The relative errors of the calculated ratios compared to the mixing ratios were less than 2%. The relationship between the average peak area ratio and mixing ratios yielded good linearity (Figure 2c) with a correlation coefficient of 0.9999 and slope value of 0.985. This agreement between expected and calculated ratios indicated that incorporation of isotopically labeled IAA to the cysteinyl residue of protein(s) during the conventional proteolytic process could be used for quantitative analysis without adding any of the preprocessing routines often necessary when using conventional isotope labeling methods.

Quantification of Lectin-Specific N-Glycopeptides with iCCM in Online mHFER-nLC-MS/MS. The potential of the iCCM method for the relative quantification of N-glycopeptides was examined using mHFER-nLC-MS/MS for online lectin-specific enrichment of CM-/iCCM-labeled N-glycopeptides using three different protein standards: AGP, fetuin, and transferrin. Two aliquots (30 μg each) of each standard protein solution were labeled with IAA and heavy IAA, respectively, according to the procedure described above with BSA. The CM- and iCCM-labeled protein standard were mixed at ratios of 5:1, 1:1, and 1:5. Each mixture was subjected to proteolysis and combined with ConA (2 μg) for complexation. Then, the mixtures of ConA and labeled peptides were loaded into the mHFER where peptides with no affinity to ConA were washed off via pores in the mHF membrane wall. After depletion of peptides nonspecific to ConA, the remaining N-glycopeptides bound to ConA (likely tetramer, ~104 kDa at pH 8) were released by feeding 5 μL of PNGase A/F (~50 units) from the autosampler into the mHFER, as shown in Figure 1, so the deglycosylated peptides could elute off the mHFER and be loaded into the RP2 trap before nLC-MS/MS. Analysis was carried out in triplicate qualitatively and quantitatively. AGP is known to have five N-linked glycosylation sites; three of those proteolytic peptides contain cysteine. Figure S2a in the Supporting Information shows the MS spectra of a pair of ConA-specific CM-/iCCM-labeled AGP peptides, QDQ**c**IY**n**TTYLNVQR (m/z 959.44, +2) and QDQ**c***IY**n**TTYLNVQR (m/z 961.45, +2), acquired by online mHFER-nLC-MS/MS analysis under three different mixing conditions. While AGP contains three N-glycopeptides with cysteine, identification of a glycopeptide, MALSWVLTVL SLLPLLEAQI PL23CANLVPVP IT33NATLDQIT GK (monoisotopic mass 4468.51 Da), was not successful in this study. Since this peptide contains four prolines, the proline effect may induce unusual fragmentation in high-energy CID experiments leading to a failure in identification. Figure S2b, Supporting Information, represents the superimposed EICs of both precursor ions (CM- and iCCM-labeled N-glycopeptides) showing no difference in average retention times between the two types (29.47 min for both). Similar MS spectra of a pair of ConA-specific CM-/iCCM-labeled peptides from fetuin and transferrin are shown in Figure S2c, Supporting Information. The calculated peak area ratios of the two labeled ions from AGP are listed in Table 1 along with other ConA-specific CM-/iCCM-labeled N-glycopeptides (one from fetuin and two from transferrin). The calculated peak area ratios at the three mixing conditions match well with the expected ratio within 3−8%

Table 1. Calculated Peak Area Ratio (n = 3) of CM-/iCCM-Labeled N-Glycopeptides in Three Glycoprotein Standards Measured by Varying the Mixing Ratio of CM-/iCCM-Labeled AGP Followed by Lectin (ConA) Affinity Enrichment and Quantification of N-Glycopeptides Using mHFER-nLC-MS/MS[a]

		m/z (charge)		calculated ratio (CM/iCCM)		
protein std.	peptide sequence identified	CM	iCCM	5 (5:1)[b]	1 (1:1)[b]	0.2 (1:5)[b]
AGP	QDQ**c***IY**n**TTYLNVQR	959.44 (+2)	961.45 (+2)	5.21 ± 0.25	1.04 ± 0.13	0.20 ± 0.01
	QDQ**c***IY**n**TTYLNVQRE**n**GTISR	892.41 (+3)	893.75 (+3)	5.09 ± 0.18	1.08 ± 0.11	0.21 ± 0.01
fetuin	L**c***PD**c***PLLAPL**n**DSR	871.38 (+2)	875.40 (+2)	5.14 ± 0.17	1.03 ± 0.05	0.21 ± 0.01
transferrin	**c***GLVPVLAENY**n**K	739.33 (+2)	741.34 (+2)	5.12 ± 0.35	1.03 ± 0.04	0.21 ± 0.01
	QQQHLFGS**n**VTD**c***SGNF**c***LFR	839.55 (+3)	842.22 (+3)	5.06 ± 0.22	1.04 ± 0.06	0.21 ± 0.01
				5.12 ± 0.11	1.04 ± 0.03	0.21 ± 0.00

[a]The asparagine site of N-glycosylation is marked **n**. [b]Mixing ratio of each labeled glycoprotein.

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differences, which are slightly larger than the differences for BSA. An explanation is that the N-glycopeptides examined in this study underwent additional experimental steps compared to the BSA peptides, such as lectin-complexation, deglycosylation, and elution through the mHF membrane, during which some unwanted loss of peptides may have occurred. Nonetheless, the concordance with expected values provided further proof of the validity of this new hyphenation method: iCCM-based isotope labeling incorporated into mHFER, an alternative tool for online enrichment of lectin-specific N-glycopeptides. The methods described so far could therefore represent an effective solution for obtaining quantitative data sets of lectin-specific N-glycopeptides.

iCCM-Based Quantification of N-Glycoproteins from Liver Cancer with Online mHFER-nLC-MS/MS. Initial evaluation of the iCCM-based relative quantification of N-glycopeptides from AGP using online mHFER-nLC-MS/MS demonstrated that both qualitative and quantitative information on lectin-specific N-glycoproteins can be obtained. The online lectin-specific quantitative approach was applied to human serum samples from liver cancer patients and a pooled healthy control to confirm its usefulness in quantitative profiling of N-glycopeptides in large scale protein samples. A 1:1 mixture of control (CM-labeled) and liver cancer (iCCM-labeled) proteome samples was digested with Lys-C/trypsin, and the resulting CM-/iCCM-labeled peptide digests were mixed with lectins. In this case, two different lectins (AAL for the selective isolation of core-fucosylated glycopeptides and ConA for α-D-mannosylated or α-D-glucosylated ones) were used individually. Online enrichment and quantification of AAL- or ConA-specific N-glycopeptides of sera were the same as those described for AGP. mHFER-nLC-MS/MS analysis of CM-/iCCM-labeled serum peptide mixtures yielded an identification of 73 CM-/iCCM-labeled N-glycopeptide pairs from both lectins, which belonged to 49 N-glycoproteins as listed in Table S2 (Supporting Information). Most N-glycopeptides were identified from both CM-/iCCM-labeled peptide mixture samples; the exception was three N-glycopeptides that were not identified from the mixture sample treated with AAL. Seventy-three N-glycopeptides were subjected to MRM analysis to quantitatively profile the liver-cancer-specific N-glycopeptides. For the MRM analysis, we first screened the individual MS/MS spectra of the 73 CM-/iCCM-labeled N-glycopeptide pairs by HCD in nLC-MS/MS to sort out quantifier fragment ions. Then, targeted MRM experiments with nLC-MS/MS were carried out for all 73 CM-/iCCM-labeled deglycosylated peptide pairs based on inclusion lists containing m/z values for each precursor and the quantifier ions.

Figure 3a shows the base peak chromatogram of the CM-/iCCM-labeled deglycosylated peptide mixture sample from control/liver cancer sera using targeted MRM experiments. In this case, the labeled peptide mixtures were treated with AAL for lectin-specific binding of the N-glycopeptide mixtures. Three cancer patient samples were individually labeled and analyzed against the pooled control sample. Figure 3b shows the superimposed EICs of fragment ions (combined EIC signal from dual transition factors as y_{11}^+ fragment ions (m/z 1270.65 and 1274.65) and y_{12}^+ fragment ions (m/z 1385.67 and 1389.67)) from respective precursor ions of VcQDcPLLAPLnDTR (+2, m/z 886.92) and Vc*QDc*PLLAPLnDTR (+2, m/z 890.93, both at t_r = 31.65 min) of 2-HS-glycoprotein in the control and liver cancer samples. The two MS/MS (HCD) spectra of CM-/iCCM-labeled pairs were plotted in Figure 4

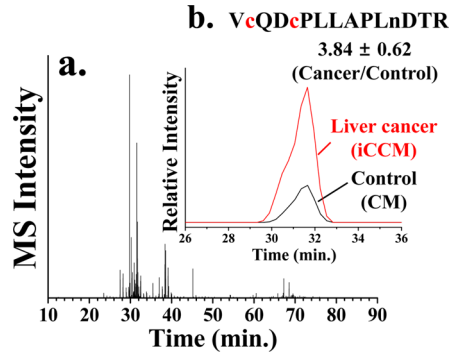

Figure 3. (a) MRM chromatogram of AAL-specific CM-/iCCM-labeled N-glycopeptides after online enrichment and deglycosylation of glycopeptides by mHFER-nLC-MS/MS: control (CM-labeled) and liver cancer (iCCM-labeled); (b) superimposed EICs of fragment ions (combined from MRM transitions to y_{11}^+ and y_{12}^+ fragments) from both CM-/iCCM-labeled VcQDcPLLAPLnDTR (from Alpha-2-HS-glycoprotein) extracted from each MS/MS spectrum.

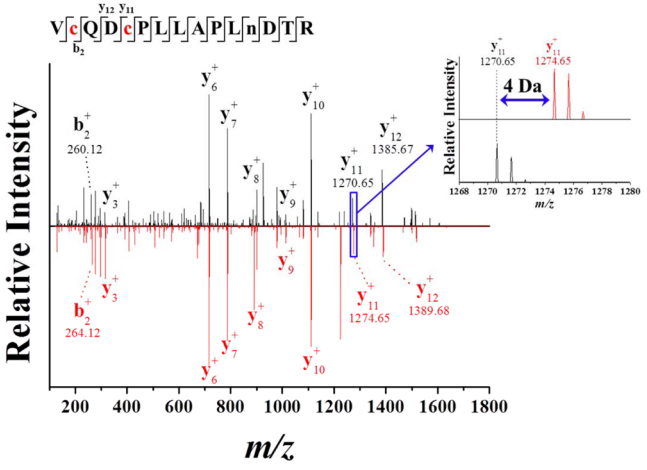

Figure 4. MS/MS spectra (HCD) of a CM-(upward)/iCCM-labeled (downward) peptide, VcQDcPLLAPLnDTR (m/z 886.92/890.93, t_r= 31.65 min) from α2-HS-glycoprotein, showing differences in the m/z values of fragment ions depending on the presence of Cys.

with up/down orientations, respectively, representing a clear difference in observed m/z values for fragment ions containing cysteine residue: 4 Da difference between corresponding fragment ion pairs, b_2^+ (m/z 260.12 vs 264.12), y_{11}^+ (m/z 1270.65 vs 1274.65), and y_{12}^+ (m/z 1385.67 vs 1389.68). Therefore, quantitative analysis of this peptide pair was made by comparing the combined peak area of the y_{11}^+ and y_{12}^+ fragment ions from dual MRM transitions of m/z 886.92 → m/z 1270.65 (y_{11}^+) and →m/z 1385.67 (y_{12}^+) from the CM-labeled precursor ion and dual MRM transition of m/z 890.93 → m/z 1274.65 (y_{11}^+) and →m/z 1389.67 (y_{12}^+) from the iCCM-labeled one. As a result, the level of fucosylation of peptide VcQDcPLLAPLnDTR from α2-HS-glycoprotein was observed to be 3.84 ± 0.62-fold more abundant in sera from liver cancer patients than from controls. While AAL-based enrichment of N-glycopeptide resulted in a significant quantitative difference (3.84-fold) in the relative amounts of the peptide VcQDcPLLAPLnDTR from α2-HS-glycoprotein, treatment of the same CM-/iCCM-labeled proteome samples with ConA followed by mHFER-nLC-MS/MS analysis with MRM exhibited no significant differences (0.79 ± 0.25-fold), as listed in Table S2, Supporting Information. This contrast

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Table 2. A Total of 19 N-Glycopeptides (from 14 N-Glycoproteins) Showed More than a 2.5-Fold Change in Their Levels in Liver Cancer Patient Samples Compared to the Control, Obtained by Online Lectin-Affinity-Based Enrichment of CM-/iCCM-Labeled N-Glycopeptides and MRM Quantitation Using mHFER-nLC-MS/MS

identified glycoproteins			identified N-linked glycopeptides				
				m/z (charge)		ratio (cancer/control)	
accession	description	MW (kDa)	peptide sequence	CM	iCCM	AAL-specific	ConA-specific
P02765	α2-HS-glycoprotein	39.3	VcQDcPLLAPLnDTR	886.92 (+2)	890.93 (+2)	**3.84 ± 0.62**	0.79 ± 0.25
			VcQDcPLLAPLnDTRVVHAAKAALAAFNAQNnGSNFQLEEISR	1575.32 (+3)	1578.10 (+3)	**3.38 ± 1.82**	**0.30 ± 0.14**
P02763	α1-acid glycoprotein 1	23.5	QDQcIYnTTYLNVQR	959.44 (+2)	961.45 (+2)	**4.57 ± 0.18**	0.75 ± 0.19
			QDQcIYnTTYLNVQREnGTISR	892.41 (+3)	893.75 (+3)	**10.89 ± 6.56**	0.47 ± 0.02
P04220	Ig mu heavy chain disease protein	49.3	GLTFQQnASSMcVPDQDTAIR	1171.04 (+2)	1173.04 (+2)	**6.58 ± 2.52**	2.25 ± 0.23
			THTnISESHPnATFSAVGEASIcEDDWNSGER	1173.84 (+3)	1175.17 (+3)	**2.59 ± 0.21**	2.32 ± 0.16
P27169	serum paraoxonase/arylesterase 1	39.7	SLDFNTLVDnISVDPETGDLWVGcHPNGMK	1110.86 (+3)	1112.19 (+3)	**0.08 ± 0.02**	0.67 ± 0.09
P00450	ceruloplasmin	122.1	AGLQAFFQVQEcnK	820.90 (+2)	822.90 (+2)	**3.45 ± 0.39**	1.20 ± 0.25
P01042	kininogen-1	71.9	ITYSIVQTncSKENFLFLTPDcK	1390.17 (+2)	1392.17 (+2)	**3.19 ± 0.20**	0.47 ± 0.13
P04196	histidine-rich glycoprotein	59.5	IADAHLDRVEnTTVYYLVLDVQESDcSVLSR	896.19 (+4)	897.19 (+4)	**0.22 ± 0.03**	1.72 ± 0.43
			VIDFncTTSSVSSALANTK	1008.48 (+2)	1010.48 (+2)	0.75 ± 0.12	1.14 ± 0.18
B7Z550	complement component 8, beta polypeptide	60.1	YAYLLQPSQFHGEPcnFSDK	801.37 (+3)	802.70 (+3)	**3.08 ± 2.10**	1.05 ± 0.26
P05543	thyroxine-binding globulin	46.3	VTAcHSSQPnATLYK	559.93 (+3)	561.27 (+3)	**8.01 ± 1.63**	1.66 ± 0.53
P04278	sex hormone-binding globulin	43.8	SHEIWTHScPQSPGnGTDASH	769.32 (+3)	770.66 (+3)	**8.39 ± 1.82**	1.50 ± 0.74
Q6LAM1	heavy chain of factor I (fragment)	35.9	FLNnGTcTAEGK	656.79 (+2)	658.79 (+2)	**0.05 ± 0.01**	1.31 ± 0.19
Q04756	hepatocyte growth factor activator	70.6	cQIAGWGHLDEnVSGYSSSLR	779.70 (+3)	781.03 (+3)	**0.32 ± 0.08**	**0.24 ± 0.09**
Q9UGM5	fetuin-B	42.0	GcnDSDVLAVAGFALR	833.40 (+2)	835.40 (+2)	0.53 ± 0.08	0.49 ± 0.21
			VLYLAAYncTLRPVSK	623.67 (+3)	625.00 (+3)	**0.23 ± 0.21**	**0.30 ± 0.02**
Q96IY4	carboxypeptidase B2	48.4	AHLnVSGIPcSVLLADVEDLIQQQISNDTVSPR	1197.60 (+3)	1198.94 (+3)	**5.90 ± 0.51**	1.02 ± 0.02

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suggests that the increase in the level of α2-HS-glycoprotein in liver cancer patients might originate from an increase in fucosylation rather than in total protein level. Moreover, it shows that the combination of two lectin treatments provided different information about the glycan environment at the same glycosylation site depending on the level of fucosylation. Although several glycopeptides in Table S2, Supporting Information, showed similar ratios (cancer/control) in both AAL- and ConA-treated samples, few species exhibited significant differences, as described with the peptide in Figure 3b. For the MRM quantification in Table S2, Supporting Information, dual transitions were used for each peptide. Among the 73 N-glycopeptides (from 49 glycoproteins) listed in Table S2, Supporting Information, 19 glycopeptides (from 14 glycoproteins) were found to be more than 2.5-fold different in the liver cancer proteome sample compared to the control (Table 2). Whereas nine glycoproteins in Table 2 showed significant increases in the level of fucosylation in liver cancer patient samples, the relative levels in ConA-specific N-glycopeptide were not significantly altered. Among them, the fucosylation level of six glycoproteins were reportedly increased for liver cancer samples: α-HS-glycoprotein,[28−30] α1-acid glycoprotein 1,[8,31] ceruloplasmin,[29] kininogen-1,[29,32] sex hormone-binding globulin,[30] and carboxypeptidase B2.[30] Moreover, serum paraoxonase/arylesterase 1 and hepatocyte growth factor activator were decreased in both AAL- and ConA-captured peptide levels (Table 2), matching well with a report showing a decrease in overall glycosylation level for liver fibrosis in hepatitis C patients[33] and liver cancer patients,[34] respectively. Ig mu heavy chain disease protein[34] and thyroxine-binding globulin[35] were reported with an overall increase in glycosylation level, matching well with our results. Moreover, our results supported that the increase in the level of N-linked glycosylation of the last two targeted glycoproteins might originate from an increase in fucosylation levels (6.58- and 8.01-fold, respectively).

CONCLUSIONS

Evaluations of the iCCM-based quantitation method with protein standards demonstrated its simplicity, accuracy, and robustness in protein level quantitation, and the combination of the iCCM method with mHFER-nLC-MS/MS made possible online lectin-specific enrichment of N-glycopeptides with quantitative proteomic analysis. Because the iCCM method is based on a typical protection of thiol groups with CM, which has been routinely used before proteolysis, it does not require special reaction skills or additional precautions for purification of labeled products. Several advantages of the CM-/iCCM-labeling method include: proteolysis after mixing two differently labeled proteins can bypass the chances of unequal digestion in two different protein samples and of unequal retrieval of peptides when two individual digestion and purifications take place before isotope labeling, as in other isotope labeling methods. Because CM-/iCCM-labeling takes place at the protein level, this method reduces a purification step to remove reagents found in typical isotope labeling reactions. Moreover, the light and heavy IAA reagents required for CM are much cheaper than typical isotope labeling reagents, which make them more accessible. The use of mHFER prior to nLC-MS/MS provides another merit by enriching CM-/iCCM-labeled glycopeptides within a porous hollow fiber membrane without a lectin-immobilized or special column.

Although the current iCCM-based quantification with mHFER-nLC-MS/MS provides the advantages described above, it cannot quantify N-glycopeptides without a Cys residue. However, the iCCM method coupled with online mHFER-nLC-MS/MS reduces sample complexity in an online process by using lectin-based enrichment of N-glycopeptides, which can be useful for the quantitation of targeted N-glycoproteins. The present study with serum proteome samples demonstrated its ability to quantify 73 N-glycopeptides (from 49 N-glycoproteins), among which 19 N-glycopeptides from 14 N-glycoproteins showed more than a 2.5-fold aberrant change in abundance in sera from liver cancer patients. The relatively low numbers of glycoproteins quantified by the iCCM method can be improved by utilizing specific antibodies to capture glycopeptides instead of lectins.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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