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Development of a parallel microbore hollow fiber enzyme reactor platform for online ^{18}O-labeling: Application to lectin-specific lung cancer *N*-glycoproteome

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ABSTRACT

We introduce a simple online ^{18}O-labeling protocol for protein samples that uses a parallelizing microbore hollow fiber enzyme reactor (mHFER) as an alternative tool for online proteolytic digestion. Online ^{18}O-labeling is performed by separately attaching two mHFERs in parallel to a 10-port switching valve with a high-pressure syringe pump and two syringes containing ^{16}O- or ^{18}O-water. ^{16}O-/^{18}O-labeled peptides are formed in this manner and simultaneously analyzed online using nanoflow liquid chromatography-tandem mass spectrometry (nLC-MS/MS) without any residual trypsin activity. The usefulness of a parallel mHFER platform (P-mHFER) in ^{18}O-labeling was tested using both cytochrome C and alpha-1-acid-glycoprotein to verify the incorporation level of two ^{18}O atoms into tryptic peptides and to provide a quantitative assessment with varied mixing ratios. Additionally, our ^{18}O-labeling approach was used to study the serum *N*-glycoproteome from lung cancer patients and controls to evaluate the applicability of lectin-based quantitative *N*-glycoproteomics. We successfully quantified 76 peptides (from 62 *N*-glycoproteins). Nineteen of these peptides from lung cancer serum were up-/down-regulated at least 2.5-fold compared to controls. As a result, the P-mHFER-based online ^{18}O-labeling platform presented here can be a simple and reproducible way to allow quantitative proteomic analysis of diverse proteome samples.

1. Introduction

In quantitative proteomics, a platform for stable isotope-labeling with advanced mass spectrometry (MS) has widely served as a fundamental way to both identify and quantify protein(s) of interest. This is achieved by simultaneously searching tandem MS (MS/MS) spectra of proteolytic peptides against a proteome database and directly measuring the relative abundance ratios of MS or MS/MS intensities for the corresponding ion pairs labeled with light−/heavy-isotope tracers [1,2]. These MS- or MS/MS-based quantitative analyses provide important clues for understanding the biological functions or metabolic pathways of targeted protein(s) in a cell, and they also reveal diverse virulence factors related to human diseases [3,4].

In contrast to chemical isotope-labeling methods (e.g., TMT [5,6], mTRAQ [7], and iTRAQ [8,9]) used in general quantitative proteomics, ^{18}O-labeling is easily achieved using the hydrolytic reaction during general proteolysis without additional sample treatments or extra reagents [10,11]. It involves incorporation of two ^{18}O atoms into the carboxyl-terminus of proteolytically cleaved peptides in ^{18}O-water, leading to a mass difference of 4 Da between the ^{18}O-labeled and ^{16}O-labeled peptides digested with ^{16}O-water. Despite its usability, ^{18}O-labeling has been used less frequently for quantitative proteomics because it is time consuming (~24 h for direct labeling or ~48 h for decoupled labeling) and labor-intensive, and back exchange occurs (from ^{18}O to ^{16}O). Back exchange, which might be caused by either residual trypsin activity or reaction with ^{16}O from the surroundings, is a particularly significant detriment to obtaining reproducible datasets for protein quantification, because quantitative analysis in ^{18}O-labeling

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involves direct measurement of the relative abundance ratios of ^{16}O-labeled peptide(s) to ^{18}O-labeled one(s) from corresponding protein pairs. Recent attempts, including heating at > 80 °C [12], pH reduction to < 2 [13,14], use of on-/off-line immobilized enzyme reactors (IMERs) [15,16], high-intensity focused ultrasound (HIFU)-IMER [17], and on-line pressured digestion systems [18], have improved the reproducibility, rapidity, and automation of quantitative proteomics.

Recently, we introduced a molecular weight (MW) sorting-based enzyme reactor, termed a microbore hollow fiber enzyme reactor (mHFER), for online proteolytic digestion and selective enrichment of lectin-specific *N*-glycoproteins. Fabrication of the mHFER module and its systematic operation for online proteolytic digestion for next nano-flow liquid chromatography-tandem mass spectrometry (nLC-ESI-MS/MS) experiments are detailed in our previous studies [19,20]. Briefly, protein samples and trypsin are sequentially introduced into the micro-volume mHFER (approximately 10 μL) via an autosampler with a continuous microflow (approximately 5 μL/min) for proteolytic digestion on the inside of a hollow fiber (HF) membrane. The digested peptides that are smaller than the HF MW cut-off (10 kDa) are simultaneously passed through an online reverse-phase (C18) trapping column for the subsequent shotgun analysis. The MW sorting-based online enzyme reactor, similar to previously reported IMERs, has the benefit of separating eluted digests from free trypsin (23.8 kDa; so it does not pass through the HF pores), thereby reducing the chance of back-exchange caused by residual trypsin activity. It also allows rapid digestion of protein samples in a short time (approximately 30 min), because of the gradually increasing trypsin-to-protein mass ratio (w/w).

To evaluate the potential of a mHFER in proteolytic ^{18}O-labeling, the present paper introduces an online parallel mHFER (P-mHFER) platform that enables a separate feeding of ^{16}O-water and ^{18}O-water into each of two reactors attached in parallel to a 10-port switching valve with a high-pressure dual syringe pump. This configuration allows the formation of ^{16}O-/^{18}O-labeled peptides and an online concentration onto a trapping column for the next shotgun analysis without any residual trypsin activity. Both cytochrome C (12.4 kDa) and alpha-1-acid-glycoprotein (AGP, 23.5 kDa), as a protein standard, were used to verify the efficiency of incorporation of two ^{18}O atoms into tryptic peptides and to evaluate back exchange, as well as to determine the accuracy of quantitative measurements by varying the mixing ratios. Additionally, we applied this new P-mHFER platform toward lectin-specific quantitative *N*-glycoproteomics of serum from lung cancer patients and controls.

2. Materials and methods

2.1. Materials and chemicals

Ammonium bicarbonate (NH_4HCO_3, ABC), dithiothreitol (DTT), iodoacetamide (IAA), L-cysteine, formic acid, peptide-*N*-glycosidase F/A, and two protein standards [cytochrome C and AGP] were purchased from Sigma (St. Louis, MO, USA). Sequencing grade trypsin was obtained from Promega Corp. (Madison, WI, USA). HPLC-grade acetonitrile (ACN) and water from Burdick & Jackson (Ulsan, Korea) were used for a binary nLC separation of tryptic digests. For online ^{18}O-labeling with a P-mHFER, ^{18}O-water (97%) was purchased from Cambridge Isotope Laboratory (Andover, MA, USA). A Qproteome total glycoprotein kit with immobilized *concanavalin A* (Con A) and *wheat germ agglutinin* (WGA) was obtained from QIAGEN (Valencia, CA, USA) for enrichment of *N*-glycoproteome from lung cancer and control sera. Fused-silica capillaries (25, 50, 75, and 100 μm-i.d., 360 μm-o.d.) for the LC column and tubing connections were obtained from Technology LLC (Phoenix, AZ, USA). The 10-/6-port switching valves used for parallelizing two mHFERs, two manual injectors, fittings, adapters, and PEEK tubing were purchased from Upchurch Scientific® (Oak Harbor, WA, USA) or IDEX Health & Science LCC. The mHF consisted of polysulfone (surface-modified hydrophilic polysulfone) obtained from Kolon Central Research Institute (Yongin, Korea).

To examine the potential of online ^{18}O-labeling with a P-mHFER in the relative quantification of serum *N*-glycoproteome, a lectin-immobilized spin column was used to enrich the lectin-specific *N*-glycoproteins from three lung cancer serum samples and a pooled healthy control, as detailed in the Supporting information.

2.2. Fabrication of the mHFER

Fabrication of the mHFER used in this study to construct the P-mHFER is detailed in the Supporting information and in our previous paper [19].

2.3. The parallel mHFER system

The P-mHFER platform for online ^{18}O-labeling consisted of a high-pressure dual syringe pump (Nexus 3000) from CHEMYX (TX, USA) that was filled with the reducing reagent (50 mM ABC, 10 mM DTT; hereafter, R-buffer) dissolved in either ^{16}O-water or ^{18}O-water and connected separately to two manual injectors by capillary tubing (100 μm-i.d., 360 μm-o.d.) with both a male nut (1/16 in.-o.d.) and a 360 μm-o.d. Teflon tubing sleeve. Unless otherwise stated, the same fittings and capillary tubing were used for fabricating the P-mHFER and coupling it online to the nLC-ESI-MS/MS. The outlet capillary tubing from both injectors was plugged separately into the opposite ports (1 and 6) of a 10-port switching valve (left side of Fig. 1). The two mHFERs were placed on the 10-port switching valve at ports 10 and 2 for the inlet and outlet of one mHFER, respectively, and ports 7 and 5 for the other mHFER. The temperature of mHFER module during online tryptic digestion was controlled by a column heater from Thermo Fisher Scientific Inc. (Waltham, MA, USA). This allowed separate introduction of protein samples and ^{16}O-water or ^{18}O-water at a flow rate of 3 μL/min into each mHFER. The online nLC-ESI-MS/MS analysis was then conducted by eluting the resulting digests from each mHFER and passing them through a MicroTight adapter (P-770) with a dual lumen sleeve (F-235, 1/16 in.-o.d.) and 360 μm-o.d. capillary tubing for subsequent online concentration and desalting on a trapping column (5 cm length × 100 μm-i.d.; bead length approximately 1 cm). The tubing was packed in our laboratory with 5 μm 200 Å Magic C18AQ (Michrom Bioresources, Inc., Auburn, CA, USA). After online ^{18}O-labeling via the P-mHFER, the ^{18}O-labeled digests and their counterparts (^{16}O) retained on the trapping column were automatically fed into an analytical column by shifting the positions of the 6-port switching valve (shown as dotted lines in Fig. 1) and separated by binary gradient RPLC for MS/MS analysis. During the nLC-ESI-MS/MS analysis, the P-mHFER system was flushed by switching the flow pathways with the 10-port switching valve (left side of Fig. 1) to the initial condition to wash out the used trypsin or undigested protein in preparation for the next ^{18}O-labeling.

2.4. mTRAQ

The mTRAQ reagent, a non-isobaric labeling reagent, was applied to both protein standards of cytochrome C and AGP, to be compared to that of the quantitative results of P-mHFER-based ^{18}O-labeling and detailed in the Supporting information.

2.5. nLC-ESI-MS/MS

The efficiency of the P-mHFER for online ^{18}O-labeling was determined using the configuration of an nLC-ESI-MS/MS that is shown in Fig. 1, and details are in the Supporting information.