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Online coupling of hydrophilic interaction/strong cation exchange/reversed-phase liquid chromatography with porous graphitic carbon liquid chromatography for simultaneous proteomics and *N*-glycomics analysis

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ABSTRACT

In this study we developed a fully automated three-dimensional (3D) liquid chromatography methodology—comprising hydrophilic interaction separation as the first dimension, strong cation exchange fractionation as the second dimension, and low-pH reversed-phase (RP) separation as the third dimension—in conjunction downstream with additional complementary porous graphitic carbon separation, to capture non-retained hydrophilic analytes, for both shotgun proteomics and *N*-glycomics analyses. The performance of the 3D system alone was benchmarked through the analysis of the total lysate of *Saccharomyces cerevisiae*, leading to improved hydrophilic peptide coverage, from which we identified 19% and 24% more proteins and peptides, respectively, relative to those identified from a two-dimensional hydrophilic interaction liquid chromatography and low-pH RP chromatography (HILIC–RP) system over the same mass spectrometric acquisition time; consequently, the 3D platform also provided enhanced proteome and protein coverage. When we applied the integrated technology to analyses of the total lysate of primary cerebellar granule neurons, we characterized a total of 2201 proteins and 16,937 unique peptides for this primary cell line, providing one of its most comprehensive datasets. Our new integrated technology also exhibited excellent performance in the first *N*-glycomics analysis of cynomolgus monkey plasma; we successfully identified 122 proposed *N*-glycans and 135 *N*-glycosylation sites from 122 *N*-glycoproteins, and confirmed the presence of 38 *N*-glycolylneuraminic acid-containing *N*-glycans, a rare occurrence in human plasma, through tandem mass spectrometry for the first time.

1. Introduction

Tandem mass spectrometry (MS/MS) has become a vital and powerful analytical tool for highly sensitive, high-throughput proteomics studies of complex biological systems [1]. The complexity and wide dynamic range of protein abundances and the sheer number of proteins in biological samples have made the identification of full proteomes from a single experiment a considerable scientific challenge—partly because the finite scanning speed and dynamic range of mass spectrometry (MS) can result in under-sampling problems. The abundances of the tens of thousands of peptides that can result after protein digestion can span over 10 orders of magnitude [2]. The proteome coverage can be improved using various approaches, including sub-cellular compartment analysis, repetitive analysis of samples, data-dependent acquisition, and advanced sample separation techniques, such as the combination of two or more dimensions of liquid chromatography (LC). Multi-dimensional liquid chromatography (MDLC) can be used to expand the separation spaces to overcome the limited peak capacity of one-dimensional liquid chromatography, which utilizes only a single type of column chemistry [3]. The coupling of MDLC with MS/MS has become an indispensable technique in MS-based proteomics. Most MDLC systems at present are based on two-dimensional

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(2D) LC to enhance the chromatographic resolving power for peptide separation, thereby minimizing peptide co-elution and ion suppression during downstream electrospray MS/MS analyses. A prime example of a 2DLC system frequently employed for shotgun proteomics combines strong cation exchange (SCX) with reversed-phase (RP) chromatography (SCX–RP) to separate peptides based mainly on their charge and hydrophobicity [4].

Among the various possible MDLC systems, the combination of hydrophilic interaction liquid chromatography and low-pH RP chromatography (hereafter simplified as "HILIC–RP") offers higher resolution in the first-dimension HILIC column [5] as well as greater orthogonality than that of conventional 2D SCX–RP LC [3]. These features arise because two different separation modes operate in the HILIC–RP platform: mainly hydrophilic partitioning, but also dipole–dipole, hydrogen bonding, and weak electrostatic interactions, in the HILIC column, and separation based on peptide hydrophobicity in the second-dimension RP LC column. These two modes lead to increased analytical performance and an increased diversity of chromatographic modalities and separation chemistries [3,6]. SCX separation is based on the charges of the peptides. It is well established that multiply charged tryptic peptides typically carry no more than five charges, with doubly and triply protonated ions predominating (>80% in total). Thus, SCX separation, especially when using a salt gradient, has limited resolution, leading to peptide spillover and uneven peptide distributions across successive fractions in the first dimension of the 2D SCX–RP system [3,7,8]. HILIC–RP processes have been employed mainly offline, involving fraction collection from the first dimension and subsequent re-injection onto the next chromatographic dimension. Offline HILIC–RP offers the advantage of being simple to implement, as well as flexibility. The major drawbacks of the offline approach to MDLC are its labor-intensity, tedious sample manipulations, and potential for sample losses. Online coupling would be attractive because it allows automated and unattended analyses of samples of very small sizes with minimal sample losses. The development of online HILIC–RP systems has been difficult, however, because of solvent incompatibility between the HILIC and RP dimensions, as well as the problem of peptide dissolution in the high content of organic solvent in the HILIC starting gradient [9]. The high content of organic solvent that elutes from the first-dimension HILIC column would cause flow-through of the peptides retained in the second-dimension RP column, leading to sample loss in the void volume. Our group recently developed an online 2D HILIC–RP LC system for proteomics profiling, positioning an RP trap column before the first-dimension HILIC column to overcome the problem of sample dissolution in the highly organic buffer used for HILIC separation, while mitigating solvent incompatibility between the two online LC dimensions through use of a solvent mixing loop in addition to an SCX trap column [10]. The separation efficiency of 2DLC remains inadequate, however, when probing highly complex samples, such as trypsinized proteins of total cell lysates. Three-dimensional liquid chromatography (3DLC), which incorporates an additional dimension of separation, can provide higher peak capacities—a very desirable feature for the separation and identification of complex peptides [11–13]. Herein, we selected a combination of liquid chromatographic modalities—hydrophilic interaction chromatography, strong cation exchange fractionation, and conventional low-pH RP chromatography—that separate peptides according to different principles. We describe an online 3D HILIC–SCX–RP platform that we have operated in conjunction downstream with additional complementary porous graphitic carbon (PGC) separation to capture non-retained hydrophilic analytes. Relative to the 2D HILIC–RP system, the performance of this newly developed online MDLC platform was enhanced through the addition of the SCX column as the second LC dimension, thereby allowing additional charge separation of tryptic peptides between the first HILIC and third RP LC dimensions; each sub-fraction of peptides in the SCX column was further separated using a stepwise salt gradient, followed by low-pH RP separation in the third dimension. In short, our new strategy integrates three different prevalent peptide separation technologies (HILIC, SCX, RP) into a single online platform for peptide separation (according to hydrophilic partitioning interactions, charge, and hydrophobicity, respectively). We configured the additional PGC column after the RP trap column to trap all of the hydrophilic flow-through, thereby extending the utility of the online 3D HILIC–SCX–RP/PGC LC system to the identification of *N*-glycans (by the PGC LC component) and de-glycan peptides (by the 3D HILIC–SCX–RP LC part) in the same run. Relative to many common LC packing materials, PGC has interesting properties, including the ability to separate very hydrophilic analytes that cannot be trapped in an RP column [14]. Retention on PGC is determined by the balance between the hydrophobicity and the interactions of polarizable and polarized groups in the analytes with the PGC surface [14]. The latter interactions depend on both the nature of the functional groups, the contact area between the analytes, and the surface of the stationary phase. Because of its special retention mechanism, PGC LC can be applied in the analyses of very polar compounds, including glycans [15]. Synchronization of the fractionations between the two modules (3D HILIC–SCX–RP and PGC) provides an enabling approach for identification of both hydrophobic and hydrophilic compounds in analyses of complex mixtures, featuring a diverse range of hydrophobicities, from a single sample injection. Thus, the integrated methodology minimizes losses of non-retained hydrophilic flow-through peptides and glycans through recapturing of the hydrophilic effluent online after sample loading into the 3D HILIC–SCX–RP module for shotgun proteomics analysis. We have evaluated the qualitative and quantitative proteomics performance of this online 3D HILIC–SCX–RP/PGC system through analyses of the tryptic digests of cynomolgus monkey brain and primary cerebellar granule neurons (CGNs) using isobaric tags for relative and absolute quantitation (iTRAQ) technology [16]. We have also, for the first time, performed detailed *N*-glycoproteomics and *N*-glycomics analyses—in the same run—of cynomolgus monkey plasma using our new MDLC system with only a single sample injection event.

2. Experimental

2.1. Chemicals and materials

Protease inhibitor cocktail tablets (EDTA-free), modified sequencing-grade trypsin and *N*-glycosidase F (PNGase F) were obtained from Roche (Switzerland). iTRAQ Reagents-8plex was purchased from AB Sciex (Framingham, MA, USA). Bradford assay reagent was supplied by Bio-Rad (Hercules, CA, USA). The high-capacity multiple affinity removal column-Hu-6 (4.6 mm × 100 mm) and the reagent kit (buffers, spin filters, spin concentrators) were obtained from Agilent Technologies (Wilmington, DE, USA). Glycoprotein isolation kits, including lectin concanavalin A (Con A) and wheat germ agglutinin (WGA), were acquired from Thermo Scientific (Rockford, IL, USA). All other chemicals and protein standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hypercarb PGC packing materials (3-μm particles, 250-Å pores) were obtained from Thermo Scientific. TSKgel Amide-80 packing materials (3-μm particles, 300-Å pores) were supplied by Tosoh Corporation. Jupiter C_{18} packing materials (3-μm particles, 300-Å pores) were acquired from Phenomenex (Torrance, CA, USA). SCX packing materials (PolySulfoethyl A, 5-μm particles, 300-Å pores) were purchased from PolyLC (Columbia, MD, USA). Electronically actuated six- and ten-port, two-position

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