ISOELECTRIC FOCUSING – MULTIPLEXED REVERSED PHASE LIQUID CHROMATOGRAPHY – MALDI-TOF MASS SPECTROMETRY IN A MICROVALVE-INTEGRATED POLYMER CHIP

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ABSTRACT

A cycloolefin polymer chip supporting isoelectric focusing (IEF) and reversed-phase liquid chromatography (RPLC) is demonstrated for two dimensional peptide separations, with IEF as the first electrokinetic enrichment stage for effective fractionation of sample components prior to RPLC. The thermoplastic chip contains integrated high pressure microvalves, enabling uniform sample transfer from the IEF channel to parallel RPLC channels in gradient elution and hydrodynamic isolation between the separation dimensions. The reusable system is shown to provide efficient and rapid 2-D separations together with facile interfacing with MALDI-MS, suggesting a new route towards effective peptide analysis from complex sample matrixes.

INTRODUCTION

While mass spectrometry is a highly successful technique for proteomic identification and quantification, effective front-end separation methods is necessary to greatly enhance its analytical capability to cope with the complexity and dynamic range of proteins within biological samples. Peak capacity is an important criterion to select a proper separation method. While relatively high peak capacities can be achieved with single dimensional separation methods [1], multidimensional separations generally offer greater resolving power according to the theoretical prediction of Giddings [2].

Two dimensional polyacrylamide gel electrophoresis (2-D PAGE) is a standard multidimensional separation technique used in proteomic analysis. To overcome the intrinsic disadvantages of 2-D PAGE, including poor reproducibility, low analysis speed, and difficult automation, a variety of liquid-phase multidimensional separation techniques were demonstrated in either on-line or off-line modes. In these techniques, resolved species eluted from the first separation dimension are collected in multiple fractions and sequentially loaded into the second dimension, thus reducing analytical throughput and enhancing instrument complexity. To overcome the shortcoming of current liquid-phase multidimensional separation techniques and leverage potential benefits including small sample and reagent consumption, high analysis speed, and high levels of integration, we used microfluidic technology to develop a cycloolefin polymer (COP) chip supporting on-line isoelectric focusing (IEF) and multiplexed reversed-phase liquid chromatography (mRPLC) for high throughput two dimensional peptide separations, with IEF acting as a highly concentrating CE separation mode for effective isolation of sample components prior to mRPLC.

CHIP FABRICATION

An IEF-mRPLC chip is depicted in Fig. 1. The channel pattern was fabricated by direct micromilling of COP plaques using a computer numerical control machining system and sealed with COP cover plates in a solvent-bonding process [3]. The integrated thermoplastic chip contains two novel microvalves (Fig. 1) with maximum pressure resistance up to 24 MPa, enabling uniform sample transfer and gradient elution from each RPLC column, and ensuring hydrodynamic isolation between the

separation dimensions. Fluidic connection between the IEF-mRPLC chip and the off-chip LC pump and capillary flow splitter (Fig. 1) were established using a high pressure and low dead volume needle interface shown to withstand pressures above (40 MPa), well above the delamination limit (24 MPa) of solvent-bonded COP substrates [3] and exceeding the typical pressure limits required for efficient RPLC. Multiplexed RPLC columns were prepared by packing 5 μ m C18 silica particles to each RPLC channel, with porous polymer frits photosynthesized at column end.

Figure 1: IEF-mRPLC chip with spotting needles for MALDI-TOF MS analysis.

RESULTS AND DISCUSSION

A chip IEF and the following mRPLC separations of a fluorescein-labeled BSA digest are shown Fig. 2 and Fig. 3 respectively.

During early chip tests, the strongly hydrophobic COP channel surface was found to provide multiple non-specific

Figure 2: Focusing of Fluorescein-BSA digest in the IEF channel. (A) Sample introduction; (B) 30 min IEF of the digest.

adsorption sites for focused peptides, causing sample loss and significant decreasing IEF resolution, capacity and reproducibility. We found that HPMC can deactivate the hydrophobic surface without significantly affecting the following RPLC separations. By doping sample with 0.4% HPMC, focused peptide bands were repeatedly generated during IEF, as shown in Fig. 2 for the case of BSA digest. This figure also reveals limited dispersion of analyte into the constrained channels linking the RPLC columns, presumably due to electrokinetic mobilization from fringing fields at the channel junctions. However, the amount of the trapped samples was much smaller than that of the focused analytes in the IEF channel, and significant carryover of peptides between the LC fractions was not observed.

In comparison to the 1-D μ RPLC separation, the free dye peak does not appear in the RPLC stage of the 2-D separation since the dye is not zwitterionic but negatively charged, and migrates out of the sampling region toward the anode during IEF. About 25 components can be recognized in 1D μ RPLC separation, compared with 66 in the IEF-mRPLC separation, corresponding to a 2.6-fold improvement in overall peak yield within the same separation time frame. Using unlabeled BSA digest as a model system, MALDI-MS analysis was performed on a set of 6 sequential fractions collected from the spotting needles inserted at each end of the LC channels (Fig. 1). Comparing the results obtained with and without the front-end separation, 48 unique digest peptides were identified following IEF-mRPLC separations, while only 18 unique peptides were identified by direct MALDI-MS detection of the whole digest.

Figure 3: μ RPLC and μ IEF-mRPLC of 0.25 mg/mL Fluorescein-BSA digest. Identical RPLC conditions were used in both separations.

Data obtained from the IEF-mRPLC fractions were converted to a set of 2-D cluster maps to show the distribution of the 30 highest intensity peptides (Fig. 4). The cluster maps reveal that the majority of peptides were confined to specific channels and fractions, with little overlap across multiple MALDI target spots. For several peptides which appear in multiple target spots, these

peptides generally exhibit discrete patterns across adjacent spots, suggesting the presence of isomers for these peptides. These results reveal that the IEF-mRPLC separations are effective at isolating peptides to specific fractions defined by their pI and hydrophobicity, thereby simplifying the content of each individual fraction while preventing unwanted overlap of peptides across multiple fractions.

Figure 4: Locations of top 30 peptides identified from BSA digest by mRPLC fraction and channel. Sequential fractions were collected every 6 min over a 36 min period. Peptide m/z values are shown in the lower left of each panel. Increasing color saturation represents higher signal intensity. Mass tolerance: ± 1 Da, m/z cut-off: 500 Da.

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