

# Combining Microfluidic HPLC Chip Technology with a Hybrid Triple Quadrupole Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (QFT-12)

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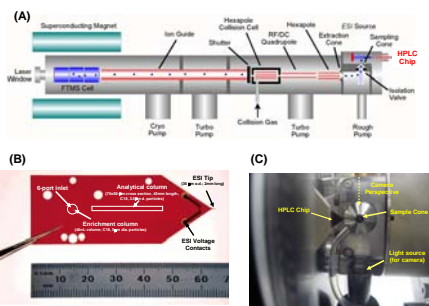
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## INTRODUCTION

- The microfluidic HPLC chip integrates an enrichment column, a separation column, and a nanospray tip into a single polyimide structure. This avoids many of the pitfalls (i.e., peak broadening, dead volumes) encountered in traditional nanoLC systems. These HPLC chips have been demonstrated to provide excellent sensitivity and retention time reproducibility (Yin et al., 2005; Fortier et al., 2005).
- Combined with a 12T QFT FT-ICR mass spectrometer, the HPLC chip provides high performing chromatographic separations that complement the high resolving power and mass accuracy of the FT-ICR. In addition, the QFT can perform a multitude of MS/MS techniques (e.g., IRMPD, ECD) to provide sequence information for both peptides and proteins separated by these HPLC chips.

Figure 1. (A) Schematic diagram of the QFT-12 FT-ICR mass spectrometer. (B) Close-up image detailing the organization of the components of the HPLC chip (Yin et al., 2005). (C) Photograph showing the positioning of the HPLC chip with the source region of the QFT-12.



## METHODS

### Mass Spectrometry

All experiments were performed using a QFT-12 triple-quadrupole FT-ICR mass spectrometer (IonSpec, Lake Forest, CA). Ions were electrosprayed by applying a potential of 1.9kV to the HPLC chip, and these ions were accumulated in the Q3 region for 2s. The typical resolving power observed in the mass spectra was between 75,000-100,000 in broadband mode ( $m/z$  185 – 2500). MS/MS was performed by using IRMPD.

### Microfluidic HPLC Chips

The experimental HPLC chip system (Agilent, Santa Clara, CA) (Yin et al., 2005) consists of an enrichment column and an LC column packed with either 3.5µm C18, C8, or graphitized carbon (GCC) particles, depending upon the analyte (i.e., peptide vs. intact protein). The eluent, flowing at 250 nL/min, is electrosprayed from the chip through an 8µm i.d. tip. An HP1100 quaternary pump delivered solvent during sample loading and a nanoLC 1100 pump delivered the gradient.

### Data Analysis

The data were analyzed by using the FT DocViewer and PeakHunter programs (IonSpec). MS (peptide mass fingerprinting - PMF) and MS/MS spectra were analyzed against SwissProt/Trembl protein databases (www.uniprot.org) using the Mascot search engine: 2 missed cleavages (Trypsin); fixed modification: carbamidomethyl (C), variable modification: oxidation (M)

## RESULTS

### MASS SPECTROMETRY

#### Resolving Power and Mass Accuracy

- Resolving powers achieved with FT-ICR were between 75,000-100,000 in broadband mode ( $m/z$  185 – 2500).
- Mass accuracy ranged from 1-5 ppm in MS mode (external calibration) and from 5-15 ppm in MS/MS mode (external calibration)

### C-18 HPLC CHIP

#### Sensitivity (Table 1)

- The HPLC chip – QFT-12 system provided good sensitivity for the PMF identification of a tryptic digest of BSA, down to 5fmol injected on-column.

Table 1.

fmol injected	# Peptides ID'd	Sequence Coverage (%)	Score
50	21	38	172
25	14	30	151
10	13	28	146
5	11	26	152

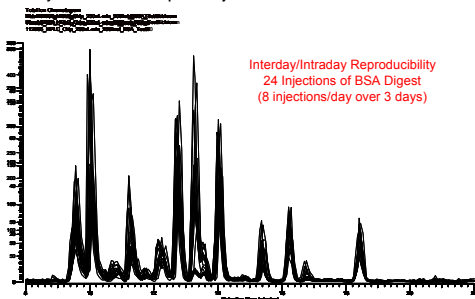
#### Retention Time Reproducibility (Table 2, Figure 2)

- Retention times (rt) for peptide ions from a standard BSA digest displayed RSDs of <1% (less than the duty cycle of these experiments).
- Analogous experiments performed with a standard C-18 nanocolumn and enrichment column yielded RSDs from 1-3%, 2-7x larger than the HPLC chip (Data not shown).
- We are currently using these data to develop new software for peptide retention time prediction and accurate mass tagging. (Conrads et al., 2000).

Table 2.

$m/z$	Amino Acid Sequence	Avg. rt / min	RSD (n=24)
571.857	KQTALVELLK	9.93	0.57%
582.317	LVNELTEFAK	10.41	0.34%
627.643	RPBFSALTPDETYVPK	9.78	0.56%
636.642	LFTFHADIBLTPDTEK	12.23	0.52%
643.269	BBAADKAEABFAVEGPK	7.13	0.55%
653.359	HLVDEPQNLK	8.19	0.74%
700.345	TVMENFVAFVDK	15.33	0.23%
710.348	SLHTLFGDELBK	10.63	0.53%
740.399	LGEYGFQNALIVR	12.73	0.42%
784.374	DAFLGSFLYEYSR	16.70	0.27%
820.471	KVPQVSTPLVEVSR	8.92	0.35%
824.402	RPBFSALTPDETYVPKAFDEK	12.19	0.29%
829.703	YNGVFQEBBQAEKGAALLPK	10.25	0.61%
862.916	MPBTEDYLSLILNR	16.82	0.27%
1002.580	LVVSTQTALA	8.91	0.27%

Figure 2. 24 overlaid chromatograms of a tryptic digest of BSA collected over 3 days. Amounts of total protein injected on-column are listed in Table 1.



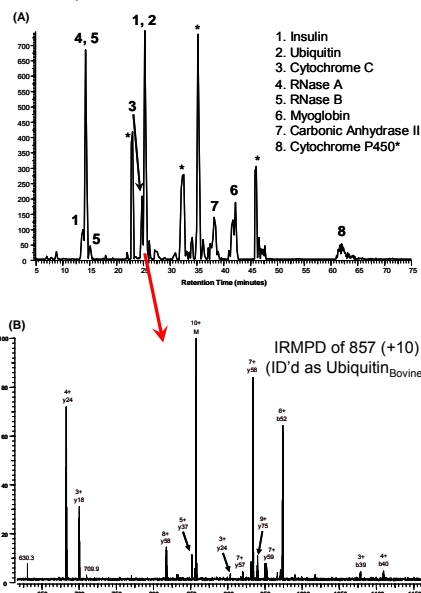
## RESULTS

### C-8 HPLC CHIP

#### Protein Mixture Analyses

- Retention time reproducibility was also excellent with the C-8 HPLC chip.
- On-column injections of less than 50 fmol/protein yielded abundant ion signals (Figure 3A).

Figure 3. (A) Total ion chromatogram of an 8-protein mixture eluted from a C-8 HPLC chip. 50 fmol of each protein was injected on-column, with the exception of 7 and 8 (125 fmol injected, each). (B) An MS/MS spectrum obtained after isolation of an ion of  $m/z$  857 (+10 charge state) in Q2, followed by IRMPD in-cell. A database search of these fragment ions yielded the correct ID: bovine ubiquitin.



### Data-dependent acquisition (DDA) of an 8-Protein Mixture

- As indicated in Figure 3, the combination of the C-8 HPLC chip with the QFT-12 enables for DDA experiments with intact proteins in a DDA-style "top-down" experiment. (Kelleher et al., 1999)
- So far, smaller proteins, such as ubiquitin (8.6 kDa) (Figure 3B) have been successfully analyzed in this way. Larger proteins may require the acquisition of several MS/MS scans, which could be afforded by manipulation of the gradient/flow rate from the C-8 HPLC chip.
- Preliminary DDA experiments employing electron capture dissociation (ECD) look promising. (Zubarev et al., 2000; Cooper et al., 2005)

## ACKNOWLEDGMENTS

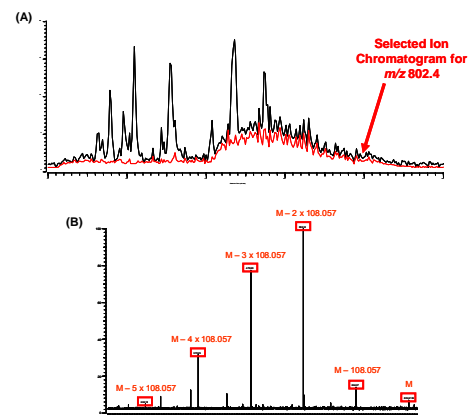
- Debbie Ritchey (Agilent) for expertise in HPLC chip manufacturing
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## RESULTS

### Contaminant Ion / Internal Calibrant

- In many of the mass spectra collected using the C-18 and GCC columns, a contaminant ion of  $m/z$  802.4 was observed.
- The presence of this ion occasionally prevented the detection of low-abundance peptides, such as during some of the analyses of 5 fmol injection of BSA digest (Figure 3).
- The appearance of this ion is potentially due to the higher current levels employed in this unique ion source interface between the HPLC chip and the QFT-12.

Figure 4. (A) Total ion chromatogram of 5 fmol BSA injected on-column on a C-18 HPLC chip. Overlaid in RED is the selected ion chromatogram for the ion of  $m/z$  802.4. (B) The MS/MS spectrum of the ion of  $m/z$  802.4, indicating repeated neutral losses of 108.057 Da.



- A combination of accurate mass data and MS/MS (IRMPD) has identified this ion as a polyacrylonitrile contaminant.
- Unfortunately, due to the ion's somewhat transient nature, the development of a more reliable internal calibration method is underway.

## CONCLUSIONS & FUTURE WORK

- The combination of the HPLC chips and QFT-12 has provided excellent sensitivity, retention time reproducibility, mass accuracy, and resolving power.
- Optimization of the MS/MS (IRMPD and ECD) and HPLC conditions required for optimal DDA experiments on intact proteins is ongoing.
- Future analyses of glycopeptides will be performed by using an HPLC chip that contains graphitized carbon columns (GCC). (Niñuovo et al., 2005)
- Development of a more reliable source of an internal calibrant is underway.

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