Prototyping of a microfluidic modulator chip and its application in heart-cut strong-cation-exchange - reversed-phase liquid chromatography coupled to nano-electrospray mass spectrometry for targeted proteomics

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Abstract

A novel multilayer modulator chip offering a robust miniaturized interface for multi-dimensional liquid chromatography has been developed. The thermoplastic microfluidic device comprises five tailor-made functional layers and the chip is compatible with commercially available switching-valve technology. The modulator chip allows for robust ultra-high-pressure operation up to 65 MPa. Peak-dispersion characteristics of system peaks were assessed directly at the valve outlet by monitoring fluorescein injection profiles with laser-induced fluorescence detection. Integration of a microporous monolithic mixing entity in the microchannels significantly narrows the resulting peak profile and depended on amount of organic modifier in the mobile phase. Proof-of-concept of the applicability of the microfluidic modulator chip is demonstrated in a heart-cut multi-dimensional strong cation exchange-reversed-phase liquid chromatography proteomics analysis workflow coupled to nano-electrospray mass spectrometry for the target analysis of Glu-1-Fibrinopeptide B spiked in a protein digest mixture of bovine serum albumin.

Keywords: Modulators, Multi-dimensional liquid chromatography, Heart-cut 2D-LC, Proteomics profiling
INTRODUCTION

The tangible benefits of multi-dimensional liquid chromatography technology with respect to realizing a high peak capacity per unit time have led to a significant momentum for two-dimensional LC (2D-LC) instrument and application development.\textsuperscript{1-13} One of the most critical elements in 2D-LC is the interface between the first-dimension (1D) and the second-dimension (2D) column, that collects and transfers fractions between developments. In comprehensive two-dimensional LC (LC\times LC), on-line modulation is typically performed using a modulator consisting of a two-position 10-port switching valve equipped with two external transfer loops, but other valve configurations have become available also.\textsuperscript{14-22} In a tutorial paper on 2D-LC, Stoll and Carr reviewed the principles of 2D-LC and showed the applicability in selected application examples.\textsuperscript{23} Pirok \textit{et al.} discussed practical considerations, including column dimensions, flow rate compatibility, and modulator-loop sizes in a review paper published in 2018.\textsuperscript{24} Generally, sampling loops with twice the modulation volume to avoid loss of 1D effluent are advised, which considers the effect of the parabolic flow profile during sampling. Ideally, sample fractions from the first development are preconcentrated in the modulator loop (packed with stationary phase) to refocus peaks prior to the 2D development.\textsuperscript{25,26} Stationary-phase-assisted modulation mediates chromatographic dilution, which effect is multiplicative in 2D-LC\textsuperscript{27,28}, and allows to optimize column dimensions targeting high loadability, applying a large i.d. 1D column, and maximum detection sensitivity utilizing a small i.d. 2D column. A generic approach to post-column analyte trapping refocusing was described by De Vos \textit{et al.}\textsuperscript{29,30} An excellent overview of possible LC modes used in on-line 2D-LC configurations, discussing solvent compatibility, was recently published.\textsuperscript{31}

Critical parameters in stationary-phase-assisted modulation include the stationary-phase chemistry in the trap column and mobile-phase composition during loading and remobilization, together defining the retention factors during the focusing and remobilization
process, before the peak is sent to the 2D column. Note, that using a trap column with an increased retention is not enough to obtain a concentration enhancement. This can only be obtained when the band is overtaken by a stronger eluent. Furthermore, peak dispersion is detrimental as bands are most sensitive to band broadening once they have been sharpened. Low plate heights are achieved using trap columns filled with small i.d. stationary phase particles and extra column dispersion induced by connection tubing should be ideally avoided. Furthermore, as is shown in Eq.(26) of De Vos et al., keeping these extra-trap volumes to a minimum, especially the volumes behind the trap column, is critical. An external loop solution inevitably always introduces a dead volume, i.e., the volume of the through-bore of the valve, between the incoming column or elution flow and the trap. After preconcentration of the fraction sampled, the external loop solution will inevitably always require the eluting peak to pass through two through-bores and one internal rotor seal groove. Using an internal trap column solution, the incoming column flow and the elution flow is in direct contact with the trap column, hence omitting the dead volume. This significantly reduces extra-column band broadening effects, which has been demonstrated in microfluidic devices.

Aiming at reducing the number of zero-dead-volume connections, making LC×LC more user-friendly while minimizing extra-column dispersion, a microfluidic modulator chip compatible with a commercial-available switching valve was designed, enabling sample transfer between columns in a 2D-LC set-up. The pressure resistance of the microfluidic chip was assessed while utilizing the valve interface, and extensive pressure stability tests were conducted in a 2D-LC modulation experiment. The dispersion characteristics of the modulator chip were determined without and with a polymer monolith present in the microchannels. Finally, the implementation of the modulator in a heart-cut SCX-RP-LC–nanoESI-MS workflow was demonstrated for the targeted analysis of a signature peptide.
EXPERIMENTAL SECTION

**Chemicals and Reagents.** HPLC grade acetonitrile (ACN) and formic acid (FA) were purchased from Biosolve (Valkenswaard, The Netherlands). Cyclohexane, (anhydrous 99.5%), fluorescein sodium salt (≥95.0%, HPLC), trizma base (≥99.9%), butyl methacrylate (99%), ethylene dimethacrylate (98%), 1-decanol (98%), cyclohexanol (99%), 2,2-dimethoxy-2-phenylacetophenone (99%), ethylene diacrylate (90%, technical grade), methyl methacrylate (contains ≤ 30 ppm mono methyl ether of hydroquinone as inhibitor, 99%), sodium chloride, sodium phosphate, phosphoric acid, trypsin, bovine serum albumin (BSA) and Glu-1-Fibrinopeptide B (Glu-Fib) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Hydrochloric acid (32%) was purchased from Merck (Darmstadt, Germany). A Pierce BSA protein digest and SMART Digest trypsin kit were acquired from Thermo Fisher Scientific (Merelbeke, Belgium).

**Microchip Design and Prototyping.** The microfluidic modulator was designed to fit a Rheodyne switching valve and comprises four intricate substrate layers of cyclic olefin copolymer (COC; TOPAS, grade 8007 Kunststoff-Zentrum Leipzig, Germany) and one substrate layer of polyether ether ketone (PEEK; Aptive 1000 series, Victrex (Lancashire, UK) designed to protect the modulator chip from shear damage caused by rotary movement. The channel layout and through-holes in the chip substrates were created using a CNC micromilling robot. Sealing of COC substrate layers was accomplished applying an optimized solvent-vapor-assisted bonding approach. Three alignment through-holes were incorporated in the chip matching the valve alignment pins, allowing the modulator chip to rotate position when the valve motor is switched. Polymer microstructures, from butyl methacrylate and ethylene dimethacrylate were synthesized in-situ in the microchannels applying UV irradiation, applying a protocol described by our group.
**Instrumentation and Operation.** Dispersion characteristics were recorded using a LED-Light Induced Fluorescence Detector (Zetalif LED 480, Picometrics Technologies SAS, Labège, France) by switching a 200 ppm fluorescein solution dissolved in TRIS buffer (pH = 8) injected in a microfluidic channel in-line with the FLD detector, meanwhile pumping with TRIS buffer at a fixed flow rate (50 µL/min). (2D-)LC-MS experiments were conducted using a Ultimate 3000 RSLC system (Thermo Fisher Scientific, Germering, Germany) coupled online with a nanoESI interface to an HCTultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The RP-LC analysis comprises a 75 µm i.d. × 150 mm Acclaim PepMap RSLC column packed with 2 µm C18 particles (Thermo Fisher Scientific) operated at a flow rate of 300 nL/min and applying a 7.5 min aqueous acetonitrile gradient (4-36 v/v% ACN) containing 0.1% FA as ion-pairing agent. BSA was digested in-house using the SMART Digest procedure. The 2D-LC heart-cut experiments were executed by performing a 1D strong cation exchange (SCX) separation, operating a 4.6 mm i.d. × 100 mm BioPro IEX SF column packed with 3 µm particles (YMC, Dinslaken, Germany) at 200 µL/min and applying a 30 min salt gradient (0-70 v/v% 0.5 M NaCl in 20 mM sodium phosphate buffer at pH 2.5) containing 25% acetonitrile. At 8.2 min, the microfluidic modulator chip was used to collect a heart-cut fraction which was directed to the subsequent 2D RP-column for LC-MS peptide mapping. TOPPView (Version 2.3.0, OpenMS package) was used for the interpretation of the data maps.

**RESULTS**

**Chip Design and Pressure Testing.** Fig. 1 shows a chip-based interface (Fig. 1A-B) designed to transfer fractions from the 1D column towards the 2D development in an asymmetrical (back-flush) configuration, see Fig. 1C. The microfluidic trap channels with a 625 nL internal volume are situated in substrate layers 4 and 5, connected to the stator ports via through-holes. Protrusions were milled at each side of the PEEK insert to firmly immobilize
the PEEK insert inside the pocket upon valve actuation. Extensive pressure-stress tests were conducted to determine the burst pressure and durability of the modulator chip. To accurately determine the average burst pressure, the flow rate was ramped increasing the operating pressure in steps of approximately 10 MPa including a 10 min hold, see Fig. S1 in the Supporting Information for comparison of pressure traces in comparison with a commercially available Tefzel rotor. Repeated burst test performed for five different modulator chips shows an average pressure resistance up to 65.4 MPa (n = 5, RSD = 6.5%). Post-experimental visual inspection using red dye showed that the microchannels remained intact and delamination was prevented, hence leak flow between PEEK insert and COC chip substrate most likely induced chip failure. The highest burst pressure applying a linear flow rate ramp-up with a rate of 1 mL/min² was determined to be 98 MPa. It should be noted that the rotor-stator interface contributes to the high pressure resistance. Fig. 2A and B display the pressure traces in a modulation experiment applying a switching frequency of 60 s, in which stress is applied to both microfluidic channels, using the LC×LC configuration depicted in Fig. 1C and maintaining the maximum operating pressure at 40 MPa. The top transfer microchannel is subjected to pressure build-up and release from 0 to 40 MPa and vice versa in seconds. At the same time, the lower microfluidic channel is switched in-line with a restrictor capillary maintaining the pressure continuously at 40 MPa. Small fluctuations in the pressure signal are caused by the brief moment (milliseconds) when the rotor switches. Following over 250 subsequent cycles, no leak flow nor delamination of the modulator chip were observed.

Dispersion Characteristics. Fig. 3 shows an overlay of dispersion profiles of fluorescein recorded directly at the chip outlet through a 50 µm i.d. capillary (<15 mm effective length) mounted on to the stator. The solid black profile in Fig. 3 shows a dispersion profile when passing though the microchannel of the microfluidic modulator chip featuring 500 × 500 µm (w × d) microchannels with 300 µm i.d. through-holes (625 nL) and stator outlet. The black
dashed overlay trace represents a dispersion profile obtained with a custom-made PEEK loop having similar volume, externally mounted on the injection valve. Peak profiles were manually shifted to coincide at their apex. Note, that the tailing peak profile is typical for short open channel layouts in pressure-driven mode, as described by Golay. The slopes of the front of the peak profiles obtained on the microfluidic modulator chip is steeper (see also Fig. S2), as the peak profile is directly passing through the stator port, whereas externally mounted loops generate more extra-column dispersion introduced by dispersion induced by dispersion contribution induced by the rotor groove and stator through holes. Dispersion characteristics were also assessed in a microfluidic chip that incorporates a polymer-based monolithic microstructure in the microchannels which can be employed to enrich and focus analytes after the 1D development prior to the 2D run. The monolith is acting as micromixer, resulting in improved longitudinal mixing of the analyte band, leading to a significant reduction of peak asymmetry and hence peak sharpening. Replacing the aqueous TRIS buffer (blue trace) for an acetonitrile-rich mobile phase (red trace) led to a further sharpening of the dispersed band due to the use of a strong remobilization solvent, as described by De Vos et al.

Heart-Cut SCX×RP-LC–MS for Targeted Analysis. The targeted analysis of Glu-1-Fibrinopeptide B (Glu-Fib, m/z = 785.8 for z = 2+) spiked in a tryptic protein digest originating from BSA was performed with nano-RP-LC-nanoESI-MS and compared with a heart-cut SCX×RP-LC–MS proteomics analysis utilizing the microfluidic modulator chip for sample transfer. The resolving power of the RP-LC analysis obtained on a 150 mm long nanoLC column packed with 2 µm particles applying a 7.5 min effective gradient was insufficient to resolve the signature peptide from BSA peptides. The 1D-RP-LC-MS experiment was performed by injecting Glu-Fib dissolved in neat mobile phase (see Figure 4A for the zoom-in of the data map) and Glu-Fib spiked to BSA digest with a concentration ratio of 1:10 (Figure 4B). The peak count of Glu-Fib, characterized by the five main isotope peaks, was reduced by
65% in the latter separation compared to the one in Figure 4A. The peak counts and the
calculation of the peak count reduction are provided in the Supporting Information. To reduce
ion-suppression effects by increasing the resolving power, a heart-cut SCX-RP-LC–MS setup
was configured, integrating a microfluidic modulator chip with an internal volume of 625 nL
to direct a target fraction containing Glu-Fib from the first-dimension SCX development and
direct it toward subsequent second-dimension RP-LC-MS analysis. By utilizing the separation
power of this heart-cut 2D-LC workflow, the signature peptide could be baseline resolved from
the other peptides, and this resulted in only 1% of peak count reduction (Figure 4D), compared
to an injection of Glu-Fib dissolved in mobile phase (Figure 4C).

CONCLUSIONS

A multilayer microfluidic modulator chip interface compatible with ultra-high-pressure
LC operation up to 65 MPa characterized by low dead volumes is an enabling technology for
chip-based 2D-LC. Proof-of-principle of its operation has been demonstrated for targeted
profiling of a signature peptide in a heart-cut SCX-RP–LC-MS experiment. Applying the
modulator chip and utilizing the resolving power of 2D-LC, allows to significantly reduce ion-
suppression effects induced by of co-eluting ions competing for ionization in the ESI compared
to a 1D-LC-MS analysis.

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Notes
The authors declare no competing financial interest.

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Figure 1. Exploded view (A) and photographs (B – front and back view) of the microfluidic modulator chip, containing two 625 nL transfer channels, that can be placed between the stator and the alignment pins in a commercially-available switching valve. The set-up of the modulator chip applied in a comprehensive 2D-LC configuration, depicting the connections on the switching valve outlets is shown in (C).
**Figure 2.** Dynamic pressure tests to determine the durability of the chip device mimicking a modulation experiment in LC×LC. 20 µm i.d. fused-silica capillaries at the outlet of the microchannels and adjusting the length of the restrictors until a backpressure of 40 MPa was achieved when pumping 50:50% (v/v) isopropanol:water at a flow rate of 0.1 mL/min. The modulation time was 60 s.
Figure 3. Peak dispersion profiles of an externally mounted transfer loop (dotted black profile) with a matching volume as a microchannel in the modulator chip device (solid black profile) measured with laser-induced fluorescence detection at the valve outlet. Also shown are the peak profiles of a modulator rotor chip integrating a polymer monolith. The blue profile was recorded with TRIS buffer for remobilization, the red profile with 80% aqueous acetonitrile.
Figure 4. Comparison of data maps recorded with 1D-RP-LC-MS injecting Glu-Fib dissolved in mobile phase (A) and a Glu-Fib-digest mixture with a ratio of 1:10 (B) and heart-cut SCX-RP-LC-MS injecting Glu-Fib dissolved in mobile phase (C) and a Glu-Fib-digest mixture with a ratio of 1:10 (D). The peak count was determined and a (m/z range from 785.7 to 787.9 m/z/ and an elution window of 1 min, see the Supporting Information.
SUPPORTING INFORMATION

Prototyping of a microfluidic modulator chip and its application in heart-cut strong-cation-exchange - reversed-phase liquid chromatography coupled to nano-electrospray mass spectrometry for targeted proteomics

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A microchip was placed in the valve and the stator was closed with a torque of 2.5 N·m. To build-up the pressure in the microchannel for the static burst-pressure test, a restrictor (650 mm long, 20 μm i.d.) was installed at the outlet of the microfluidic rotor channel. The channels were filled with 50:50% (v/v) isopropanol:water to remove air. At the start of the static burst pressure test a flow rate of 0.1 mL/min with a flow rate ramp of 1 mL/min$^2$ was applied and maintained for 10 min. Afterwards, the back-pressure over the channel is increased step-wise with approximately 10 MPa until a leak was detected. The result of the static pressure test is depicted in Fig. S1. The black trace shows the pressure signal for a commercially available Tefzel rotor seal, providing a leak-tight connection. The red traces show the performance of two modulator chips. Both chips remained pressure-stable and leak-free until 65 MPa. At a higher pressure, an off-set between pressure traces become apparent, and also liquid was visually detected at the unused ports of the stator of one of the chips. By introducing a paper towel in the port, the liquid was quickly absorbed and damped the paper, allowing for a positive observation of the leak. After completing the burst test, stator was removed from the switching valve and red dye was introduced to assess stress fractures.
Fig. S1. Static burst pressure test comparing the performance of a commercially available UHPLC rotor seal (black) with two modulator chips (red traces). The burst pressures of the modulator chip devices are marked by the black arrows.

The set-up for the dynamic pressure test (Fig 2 of the main article) is realized by installing 20 µm i.d. fused-silica capillaries at the outlet of the microchannels and adjusting the length of the restrictors until a backpressure of 40 MPa was achieved when pumping 50:50% (v/v) isopropanol:water at a flow rate of 0.1 mL/min. Next, the valve was switched every 60 s to record the pressure plots depicted in Fig. 2A and Fig. 2B in the main article.
2 Analysis of the slope of the dispersion profiles

The datapoints corresponding to the front of the dispersion profile coming from a custom-made PEEK loop (7.96 cm × 100 µm i.d., volume = 0.625 µL) installed on a commercially-available switching valve (red open circles and dashed black profile) and those of a profile coming from a modulator rotor chip (black open circles and solid black profile) were fitted. The dispersion profile of the PEEK is slightly larger than that of the rotor chip, most likely due to the i.d. tolerance of the PEEK tubing (± 12.5 µm according to the manufacturer, amounting for ± 0.175 µL). A linear regression line was used, providing excellent fitting scores close to $R^2 = 1.0$. The slope of the modulator chip was found to be 30% steeper compared to that of the commonly used PEEK-loop based set-up.

Fig. S2. Analysis of the slope of the front of the dispersion profile coming from a PEEK (external) loop installed on a commercially available switching valve (red open circles) with that coming from a modulator rotor chip (black open circles). Both systems have a matching volume of 0.625 µL.
3 Quantitation of ion-suppression effects in MS

The data in Table S1 corresponds to the MS spectra maps in Fig. 4 of the main article. These 3D-maps mark the elution window of Glu-Fib (one-minute width), including five of its main isotope peaks ranging from 785.7 to 787.9 m/z. A peptide feature is defined as the group of mass peaks when tracing the isotopic pattern across the retention time dimension, thus reconstructing the chromatographic elution profile of the peptide.

Table S1 lists the area and height of the total ion peak in the defined area along with the (mass) peak count in this range, corresponding to Fig. 4. When Glu-Fib was dissolved in mobile phase, the peptide feature was observed under all conditions (Figs. 4A and C). This was verified with the “FeatureFinderCentroided” algorithm included in the OpenMS package, v2.4.0. When Glu-Fib was added to the tryptic digest mixture of BSA, the feature recognition was impaired in case of Fig 4B. Peak counts were found to be the most reliable metric to quantify the level of ion suppression of a peptide feature in our LC-MS experiments indicating a ion-suppression effect of 65% = (1-80/228)*100% found for Glu-Fib in BSA digest compared to Glu-Fib dissolved in mobile phase.

Table S1: Total ion peak area and height, and (mass) peak count in the elution window of Glu-Fib, including five of its isotopes (m/z ranging from 785.7 to 787.9), in correspondence with the MS spectra maps shown in Fig. 4 of the main article.

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