A multidimensional liquid chromatography-tandem mass spectrometry platform to improve protein identification in high-throughput shotgun proteomics

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Abstract

A new on-line multidimensional system for sequential trapping and individual elution and separation of peptides based on their molecular weight is described. By sequentially using two chemically different trapping columns, a polymethacrylate monolith and a packed C18 one, peptides from complex samples can be on-line trapped and divided into two fractions, containing respectively mainly medium-large peptides and smaller peptides. Then, by means of two switching valves working in parallel, the two fractions were individually separated by reversed phase chromatography. The whole gradient consisted of two subgradients, with the first one dedicated to the separation of smaller peptides and the second one for the separation of larger peptides. Such configuration allowed to identify up to 1476 proteins in a standard *E. Coli* tryptic digest, with improved performance, increased average sequence coverage and reduced single unique peptide identifications compared to a conventional shotgun proteomics configuration comprising only the C18 trapping column and the analytical column.

Keywords

nanoHPLC-MS/MS; multidimensional chromatography; shotgun proteomics; monolithic trapping column; peptide fractionation

1. Introduction

Supported by remarkable technological advancements in various fields of research ranging from liquid chromatography (LC), to mass spectrometry (MS) and bioinformatics, proteomics is continuously expanding across different areas [1], including the study of fundamental biological processes, the investigation of protein expression in tissues, cells and organelles, the discovery of biomarkers, the study of animal models of diseases, just to cite some of the most relevant applications [2]. In contrast to top-down proteomics [3], bottom-up proteomics relies

on the analysis of complex peptide mixtures after enzymatic digestion of proteins by trypsin or other proteases [4]. This leads to samples of tens of thousands of peptides with a very wide dynamic range of concentrations [5]. Dealing with such samples is a problem of great complexity that requires analytical systems with very large resolving power, elevated sensitivity and selectivity. To date, mainstream platforms of analysis in the field of shotgun proteomics consist of high-efficient separation systems, often micro/nano-multidimensional LC, directly coupled to fast or ultrafast tandem mass spectrometry (MS/MS), by far the most selective detection system available, usually by means of a micro/nano-electrospray ionization (ESI) interface [6-9].

It is a matter of fact that, in this area, fundamental discoveries have kept pace with technological developments. As an important example of this concept, the use of cutting-edge technology in reversed phase (RP) gradient LC coupled to fast MS/MS through nano-ESI ionization source, has recently led Köcher *et al.* [10] to the finding that there exists a linear relation between peak capacity and the number of identified peptides in complex samples.

Despite the advent of improved and faster MS instrumentation, most proteomics studies employ data-dependent mode acquisition, for which a limited number of precursor ions can be acquired for each master scan [11]. This means that an improved peptide separation can provide increased probability of precursor acquisition during MS/MS analysis and, in turn, downstream protein identification. The first approach that can be applied to achieve this goal relies on peptide fractionation prior to MS, which is usually achieved by multidimensional off-line or on-line separation on different chromatographic columns, based on different separation mechanisms [12-18]. This approach reduces the complexity of the starting sample since a smaller number of peptides is analyzed within a single run. The other approach is based on the employment of longer gradients and/or longer columns for peptide separation (without prior

fractionation) to improve the chromatographic separation and the final protein identification by means of an increased number of acquired spectra [19-21].

Peak capacity can be greatly enhanced in two-dimensional LC (2D-LC) [22]. From a theoretical viewpoint, indeed, it has been demonstrated that the maximum peak capacity of such a system is given by the product between the peak capacity of each separation dimension [23]. For this reason, 2D-LC coupled to MS/MS is currently considered the technique offering the maximum separation efficiency and represents one of the preferred choices for bottom-up proteomics [24]. In order to reach the theoretical maximum peak capacity, the two dimensions of the 2D-LC system must be orthogonal, i.e., they have to be based on two completely different separation mechanisms [22,24,25]. Orthogonality condition is rarely if ever met. As a consequence, true peak capacity of 2D-LC systems can be significantly lower than the maximum achievable one and its value is further diminished by practical limitations, first of all band-broadening caused by system or in-column void volumes [9].

Briefly, multidimensional applications in proteomics can be off-line fractionation or direct on-line analysis workflows [26,27]. The off-line multidimensional approach is the most flexible one, where the first dimension is used to collect eluting fractions at regular time intervals, which are then further separated on the second dimension. The lack of direct coupling allows to combine chromatographies which are not directly compatible, since samples can be desalted and/or lyophilized after the the first separation. However, such an approach requires laborious sample manipulation and is more prone to potential sample loss and contamination [26,28]. In contrast, the on-line approach can be automated and enables the direct transfer of fractions generated from one dimension to the following chromatographic stage for further separation. The main advantages are the much smaller sample amount necessary than the off-line approach, the reduced sample loss and the shorter overall analysis times [29-31]. However, a significant limitation in on-line 2D-LC system interfaced to MS via ESI is that the (relatively)

elevated flow rates needed on the second separation dimension, to properly sampling the first one can be detrimental to the achievement of elevated sensitivity [32]. In this regard, a promising approach has been recently described for direct interface with the MS of comprehensive approaches for complex peptide mixture analysis [33].

Systems where one or more trapping columns are used in conjunction with a "true" separation column are also classified as on-line multidimensional techniques [34]. They can be a valuable alternative to strictly off-line and on-line multidimensional LC. In this context, we propose an innovative, simple platform of analysis for bottom-up proteomics made of two trapping columns in time sequentially connected to a packed nanocolumn coupled with MS/MS detector via nano-ESI. The two trapping columns, a polymeric methacrylate-based monolithic one [35] and a RP C18 packed column, have been chosen with the purpose of fractionating peptides into two fractions essentially depending on their molecular weight and hydrophobicity. The system is designed to permit the on-line comprehensive transfer of the sample fraction in each trapping column to the nanocolumn for separation. This operation is performed independently for the two trapping columns, firstly with the RP packed column and then with the organic-monolith. In this proof-of-concept study, we have applied this novel on-line multidimensional system (MDS) to the separation of a commercial tryptic digest of *Escherichia Coli*.

2. Materials and methods

2.1. Reagents and Materials

All chemicals, reagents and organic solvents of the highest available grade were provided by Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All solvents for nanoHPLC-MS/MS were of LC-MS grade. The fused-silica capillary tubing (0.250 mm id, with a polyimide outer coating) used to prepare the monolithic trapping column were purchased from Polymicro Technologies (Phoenix, AZ, USA). The Acclaim® PepMap100 C18 trapping

column (300 µm i.d. × 5 mm, 5 µm particle size, 100 Å pore size) was purchased from Thermo Scientific (Bremen, Germany). The MassPREP *E. Coli* Digest Standard was provided by Waters (Milford, Massachusetts, USA), and reconstituted with 0.1% HCOOH at 0.4 µg μL^{-1} concentration.

2.2. Preparation of the γ-poly-(LMA*co*HDDMA) monolithic trapping column

The polymeric methacrylate-based monolithic trapping column (TC2) was prepared as previously described [36]. Briefly, the inner surface of the capillary was activated to increase the number of silanol groups, first with 1 mol L^{-1} NaOH for 3 h at 120°C, then with 0.1 mol L^{-1} HCl for 3 h at 70°C. Then the capillary was treated with 3-(trimethoxysilyl)propyl methacrylate as source of vinyl groups to covalently bind the polymer to the silica surface. After cutting the single pre-treated capillary to 50 mm length, the polymerization step was performed inside a 60Co Gammacell irradiating the filled capillary in horizontal position at a temperature of 25°C with a total dose of 40 KGy at a dose rate of about 2 kGy/h. The polymerization mixture used to fill the capillaries consisted of 26.4% of lauryl methacrylate (LMA), 6.4% of 1,6-hexanediol dimethacrylate (HDDMA) and a porogenic binary mixture of 47.3% tert-butyl alcohol and 19.9% 1,4-butanediol (reported percentages are *v*/*v*).

2.3. Peptide separation and nanoHPLC-MS/MS analysis

Four µL *E. Coli* standard digest were separated by RP chromatography using the Dionex Ultimate 3000 (Dionex Corporation Sunnyvale, CA, USA). Samples were preconcentrated on the Acclaim® PepMap100 C18 trapping column (TC1, see Fig. 1) alone for conventional analysis experiments and sequentially on-line preconcentrated on the γ-poly-(LMAcoHDDMA) polymethacrylate monolithic trapping column (TC2) and on the Acclaim® PepMap100 C18 trapping column (TC1) for the multidimensional experiments. In either case,

125 the sample was loaded employing a premixed mobile phase ddH_2O:ACN 98:2 (*v*/*v*) containing
126 0.1% (*v*/*v*) TFA at a flow-rate of 10 µL min^{-1}. After loading, the sample was separated by RP
127 chromatography on a 25 cm long fused silica nanocolumn (25 cm × 75 µm id) packed with
128 Acclaim-C18 particles (2.2 µm particle size) and an outlet organic monolithic frit [37] (named
129 column in Fig. 1). The LC system was operated at 250 nL min^{-1} and at 25 °C. The employed
130 mobile phases for peptide separation were ddH_2O with 0.1% HCOOH (phase A) and ACN with
131 0.1% HCOOH (phase B). Different gradients were tested. Eluting peptides were analyzed by
132 high resolution MS by a LTQ Orbitrap XL (Thermo Scientific) mass spectrometer directly
133 connected to the LC system by a nanoESI ion source. Full scan and MS/MS spectra were
134 performed in the *m/z* range of 400-1800 and 60,000 Full Width at Half Maximum (FWHM)
135 resolution (at *m/z* 400) for the full scan. A data dependent mode acquisition was enabled, in
136 top 5 mode, rejecting +1 and unassigned charge states, using a normalized collision energy of
137 35%, and an isolation window of 2 *m/z*. Ion trap and Orbitrap maximum ion injection times
138 were set to 100 and 200 ms, respectively. Automatic gain control was used to prevent
139 overfilling of the ion traps and was set to 1×10^6 for full FTMS scan, and 1×10^5 ions in MS^n
140 mode for the linear ion trap. To minimize redundant spectral acquisitions, dynamic exclusion
141 was enabled with a repeat count of 1 and a repeat duration of 30 s with exclusion duration of
142 70 s. Five technical replicates were performed for each tested gradient of the conventional
143 configuration and the on-line MDS configuration.
144
145 **2.4. Database search and peptide identification**
146 Spectra, collected as raw MS/MS data files from Xcalibur software (version 2.2 SP1.48,
147 Thermo Fisher Scientific), were searched against SwissProt database with the Proteome
148 Discoverer software (v.1.3, Thermo Scientific) and the Mascot (v.2.3.2, Matrix Science) search
149 engine, using the *E. Coli* taxonomy (22983 entries). The selected proteolytic enzyme was

trypsin and up to two missed cleavages were allowed. Carbamidomethylation was set as a fixed modification, whereas methionine oxidation, N-terminal acetylation, and N and Q deamidation were set as variable modifications. The monoisotopic mass tolerance for precursor ion and fragmentation ion were set to 10 ppm and 0.5 Da, respectively. Protein identifications were accepted if at least one unique peptide was assigned. Finally, results were filtered setting "high" as minimum peptide confidence (corresponding to a false discovery rate, FDR, <1%).

3. Results and discussion

There are three main stages of analysis in a typical shotgun proteomics experiment: a) sample preparation [38]; b) chromatographic separation and MS analysis; c) bioinformatics analysis [39-41]. In this work, we focused on chromatographic separation and MS analysis which possibly represent the most critical steps, on which critically depends both the quality of spectra acquisition and the number of data that can be processed during bioinformatics analysis, thus strongly affecting the final protein identification [42]. In this regard, we developed an approach that allows not only for on-line fractionation of peptides into low and medium-high molecular weight fractions by means of different trapping columns, but also for their gradient separation with specific programs for each fraction.

Our study starts with the evaluation of the performance of a conventional nanoHPLC setup towards the characterization of a standard *E. Coli* digest to have a reference point for the successive employment of the novel on-line MDS. In the conventional configuration, a 10-port 2-position switching valve was employed, as depicted in Fig. 1. During sample loading (Fig. 1a), mobile phase A (see Experimental section for details) delivered by the loading pump is used to bring the sample to the trapping column (TC1); at the same time, the mobile phase B, delivered by the nanopump, passes through the column. After switching from loading to injection position (Fig. 1b), the phase from the nanopump passes in back-flushing mode

through the trapping column and the separation column. With this configuration, thus the sample is totally analyzed in a single run.

In order to maximize protein and peptide identifications of the *E. Coli* standard digest, two experiments were performed with the conventional system. In the first case, a 250 min long gradient (gradient A, Fig. 2a and Supplementary Material S1), whereas in the second one a much longer gradient (450 min) was employed (Gradient B, Fig. 2b). Gradient A allowed for the identification of 1160 protein groups and 6175 peptides. Gradient B, on the other hand, permitted to identify respectively about 12% more protein groups (1300 vs. 1160, see Fig. 2c) and about 10% more peptides (6779 vs. 6175, see Fig. 2d). As shown by Venn diagrams in Fig. 2c and 2d, the two gradients share a large percentage of overlap for both proteins (72%) and peptides (62%), and only small contributions are provided by each individual experiment, up to 23% for peptides and 19% for proteins, in both cases obtained for gradient B.

The scheme of on-line MDS developed in this work is reported in Fig. 3. In order to sequentially trap different peptide populations, while loading sample as in traditional way, a different valve set-up has been employed. It requires an additional multiport valve. Incidentally, we observe that such valve is usually available on instruments and in most cases left unused. In our case, a six-port 2-position valve was introduced into the system (Fig. 3).

With this configuration, the analysis can be divided into three main steps. In the first one (Fig. 3a), the 10-port 2-position switching valve connects the loading pump to the polymethacrylate monolithic trapping column (TC2) and the packed C18 trapping column (TC1) in series; the sequential loading on the two trapping columns allows the fractionation of the sample based on hydrophobicity and molecular weight (see later on), with larger peptides retained on the polymethacrylate monolithic trapping column and smaller ones trapped on the packed C18 trapping column. After loading, both valves switch (Fig. 3b). This way, the nanopump is directly connected to the C18 trapping column and smaller peptides can be eluted and separated

by a dedicated gradient. Finally, in the third step (Fig. 3c), the 6-port 2-position valve switches back to the starting position thus disconnecting the C18 trapping column and connecting the polymethacrylate monolithic trapping column to the nanocolumn. This operation is accompanied by the start of a new gradient that can be optimized for the elution of larger peptide population. Fractionation of a complex sample into small and medium-large peptide fractions, which can be individually separated in a single chromatographic run by two independent gradients gives the opportunity of simplifying the sample complexity and to improve sub-sample chromatographic resolution. Therefore, through this on-line MDS, not only peptide separation and identification is expected to be improved, but also protein identification and sequence coverage.

In the case of the on-line MDS, for the sake of comparison, as a first attempt the same gradient A previously employed on the traditional system (Fig. 1) was used for separation of both small and medium-large peptide fractions. Under these (non-optimized) conditions, the total number of protein identifications was 1273, divided into 970 proteins for the first separation and 1025 for the second one. The total number of peptides was 6037, again divided into 3414 peptides for the first separation and 3529 for the second one. As it can be noticed, these conditions essentially provided the same information given by the conventional system.

The great advantage of the on-line MDS described in Fig. 3, however, is that it allows to employ specific gradients for each single fraction. Indeed, since the on-line MDS consists of the sequential separation of two different peptide fractions, the whole gradient can be considered divided into two main subgradients, one for each fraction, which are not required to be the same. What is necessary is an intermediate equilibration step, at the end of first subgradient, to equilibrate the analytical column and condition the system for the second separation. For this reason, the second subgradient of the total optimized program (gradient C in Fig. 4) starts at higher organic solvent concentration in the mobile phase (10% B) than the first one (2% B).

225 Fig. 4 shows the chromatogram obtained by using the on-line MDS system for the separation
226 of *E. Coli* digest sample.
227 As far as molecular weight distribution is concerned, it was found that the first elution step
228 mainly provided small peptides, 70% of the identifications in this fraction being below 1500
229 Da (Fig. 5a). On the other hand, an opposite trend was found for the second elution step, where
230 small peptides (<1500 Da) were less than 30%, while almost 30% of the identified peptides
231 were larger (with molecular weight above 2000 Da). The grand average of hydropathy
232 (GRAVY) value was employed to assess the degree of hydrophobicity of the identified
233 peptides. The GRAVY value distribution pointed out that the most hydrophilic peptides
234 (GRAVY $\leq$ -1) were twice as many in the first elution step (12% *vs.* 6%). On the contrary,
235 more hydrophobic peptides (GRAVY > 0) were identified in second elution step (46% *vs* 41%,
236 Fig. 5b).
237 With respect to the conventional system, the absolute number of the total identifications was
238 significantly improved (Fig. 6a and 6b), with an additional 313 proteins identified that represent
239 about 20% of the total protein identifications. Such gain of information comes from the better
240 peptide resolving power achievable through the described on-line MDS before MS/MS
241 analysis, which minimizes ion suppression and improves ionization efficiency and data
242 acquisition [43]. In particular, it is due to the possibility of independently treat each fraction in
243 terms of gradient program (steepness and time), flow rate and temperature, allowing thus to
244 improve both the separation efficiency and MS sensitivity for each fraction of peptides. At the
245 same time, the proposed system is very straightforward to apply. The preparation of monolith
246 trapping columns does not suffer from the typical issues encountered with packed columns,
247 such as the increased difficult in preparing columns with a smaller diameter [44]. In this way,
248 the described approach provides an enhancement to single trapping columns platforms and an
249 alternative to traditional multidimensional approaches, enabling to perform a second

chromatographic separation from a complex starting mixture by means of a very convenient set-up. By exploiting the selectivity retention of larger peptides by the monolith in the trapping column, the problem of incompatibility between phases and sample dilution are easily bypasses as well as volume compatibility issues typical of comprehensive 2D-LC approaches, for which direct interface with nanoESI is still challenging. In this system, the entire platform offers a scale which is the ideal one for proteomics applications, with capillary trapping columns for peptide fractionation into two fractions and separation on a nanocolumn, thus no splitting is required for interface with the MS. Moreover, sample handling operations are not required, thus sample amounts and loss can be reduced.

4. Conclusions

In this work, a new on-line two-switching valve MDS was described, in which complex peptide mixtures can be on-line fractionated by means of sequential loading onto two different trapping columns. The first one is a polymethacrylate monolithic trappping column suitable for trapping medium-large peptides, while the second one is a commercial packed C18 trapping column. After loading, the two-switching valves allow to individually elute the loaded peptides and sequentially separate them on the same analytical column. Dedicated subgradients, specific for each peptide fraction, can be optimized to maximize protein and peptide identifications. The application of this system to the characterization of a standard tryptic digest (*E. Coli*) demonstrated that the novel on-line MDS outperforms a conventional nano-HPLC set-up permitting to increase the sequence coverage and simultaneously to reduce the number of single unique peptide identifications, which will improve protein score identification at the bioinformatics level.

The system is easy to operate (for instance, there are no solvent compatibility issues among separation dimensions or sample dilution), it allows great operational flexibility and it is fully

automated in an instrument equipped with a second switching valve and thus suitable for high-throughput applications. Given the issues which comprehensive approaches still suffer, although the proposed system cannot be compared to it, still it adds a second dimension to the typical single trapping column setup, providing a useful and easier alternative especially valuable for the analysis of complex peptide mixture and scarce samples. We believe that this simple approach can contribute to further extending the strategies of protein identification in bottom-up proteomics.

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413 **Figure captions**

414

415 **Fig. 1.** Scheme of loading (a) and injection (b) position of the 10-port 2-position valve in a
416 conventional configuration used in shotgun proteomics experiments. TC1: Acclaim®
417 PepMap100 C18 trapping column; column: 25 cm × 75 μm fused silica nanocolumn packed
418 with Acclaim-C18 particles (2.2 μm particle size). The red connection between positions 6 and
419 3 is a 30 μm × 100 mm nanoViper^{TM} connection. See Experimental section for details.

420

421 **Fig. 2.** Base peak mass chromatograms of *E. Coli* sample (1.6 μg injected) for the conventional
422 setup and a) 250 min gradient (gradient A); b) 450 min gradient (gradient B). Venn diagrams
423 displaying the distribution of the identified proteins (c) and peptides (d) between the two tested
424 gradients for conventional analysis of *E. Coli* standard digest (1.6 μg). IN red are marked the
425 gradient steps for peptide separation, whereas the other points refer to column conditioning or
426 washing and equilibration.

427

428 **Fig. 3.** Configuration of valves in loading (a), elution-1 (b) and elution-2 (c) positions. TC1:
429 Acclaim® PepMap100 C18 trapping column; TC2: the γ-poly-(LMAcoHDDMA) monolithic
430 trapping column; column: 25 cm × 75 μm fused silica nanocolumn packed with Acclaim-C18
431 particles (2.2 μm particle size). See Experimental section for details.

432

433 **Fig. 4**. Base peak mass chromatogram of *E. Coli* sample (1.6 μg injected) for the optimized
434 on-line MDS gradient (gradient C).
435 Under the optimized gradient conditions, a total of 1476 protein groups and 8030 peptides were
436 identified. Peptide populations differing in their molecular weight distribution and
437 hydrophobicity (expressed as grand average of hydropathicity, GRAVY) were recognized in
438 the two subgradients (Fig. 5a and 5b and Supplementary Material S1).

439

440 **Fig. 5**. Molecular weight (a) and GRAVY value (b) distribution for the identified peptides in
441 the sequential elution from the packed C18 trapping column (TC1) and the polymethacrylate
442 monolithic trapping column (TC2).

443

444 **Fig. 6**. Comparison between the conventional system (gradient B) and the optimized on-line
445 MDS: Venn diagrams with the distribution of the identified proteins (a) and peptides (b); radar
446 charts comparing the sequence coverage (c) and the number of unique peptides per protein (d)
447 for the five replicates from the analysis of *E. Coli* digest sample.