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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 to 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.

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

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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 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*.

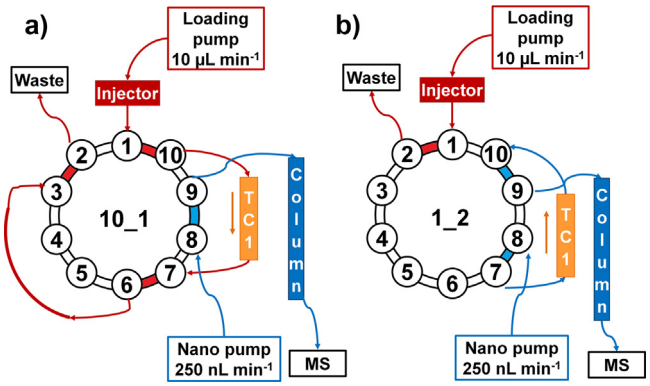

Fig. 1. Scheme of loading (a) and injection (b) position of the 10-port 2-position valve in a conventional configuration used in shotgun proteomics experiments. TC1: Acclaim® PepMap100 C18 trapping column; column: 25 cm × 75 μm fused silica nanocolumn packed with Acclaim-C18 particles (2.2 μm particle size). The red connection between positions 6 and 3 is a 30 μm × 100 mm nanoViper™ connection. See Experimental section for details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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-(LMAcoHDDMA) 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

4 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