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Short communication

Advancement in stationary phase for peptide separation helps in protein identification: Application to atheroma plaque proteomics using nano-chip liquid chromatography and mass spectrometry

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ABSTRACT

In the last decades, proteomics has largely progressed. Mass spectrometry and liquid chromatography (LC) are generally used in proteomics. These techniques enable proper separation of peptides and good identification and/or quantification of them. Later, nano-scaled liquid chromatography, improvements of mass spectrometry resolution and sensitivity brought huge advancements. Enhancements in chemistry of chromatographic columns also brought interesting results.

In the present work, the potency of identification of proteins by different nano-chip columns was studied and compared with classical LC column. The present study was applied to cardiovascular field where proteomics has shown to be highly helpful in research of new biomarkers. Protein extracts from atheroma plaques were used and proteomics data were compared. Results show that fewer spectra were acquired by the mass spectrometer when nano-chip columns were used instead of the classical ones. However, approximately 40% more unique peptides were identified by the recently optimized chip named Polaris-HR-chip-3C18 column, and 20% more proteins were identified. This fact leads to the identification of more low-abundance proteins. Many of them are involved in atheroma plaque development such as apolipoproteins, ceruloplasmin, etc.

In conclusion, present data shows that recent developments of nanoLC column chemistry and dimensions enabled the improved detection and identification of low-abundance proteins in atheroma plaques. Several of them are of major interest in the field of cardiovascular disease.

1. Introduction

Since first proteomic studies were performed, technologies and methods have been continuously improved to serve proteomics. Mass spectrometry (MS) and even tandem MS (MS/MS) based analyses were one of the major tools that brought huge advances in the field. In this context MS is often coupled to a separation technique such as liquid chromatography (LC) or capillary electrophoresis. All these techniques have also largely evolved with time. For example high resolution MS (HRMS) and nano-scaling LC enables analysis of small quantity of protein samples with high accuracy identification. Development of new multidimensional techniques including protein and/or peptide fractionation prior to LC-MS/MS analysis also brought interesting results [1]. Current proteomics might be divided into three different approaches [2]: (i) identification of proteins in complex samples called comprehensive or shotgun proteomics which is purely qualitative analysis; (ii) targeted proteomics where a specific proteins is followed in sample and

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quantified; or (iii) identification, localization and quantification of posttranslational modification(s) of a particular protein, considered as targeted proteomics.

Recent developments in nano-technologies make the use of nano-LC systems easier and bring possibilities for high-throughput analyses [3–7]. Efficiency and reproducibility of LC separation are as important as MS resolution and sensitivity for good proteomic experiments especially when samples get more complex. More than 10 years ago, Nano-LC-Chip Cube system® (Agilent Technologies, Santa Clara, CA, USA) which makes nano LC-MS analyses much easier, was developed and released. This nanoLC system includes typical instruments (autosampler, nano- and capillary-pumps) coupled to a "Cube" that includes LC-rotor switch system, nano-electrospray-ionization source, enrichment and separation columns connected to a nano-spray tip that are integrated on a single LC-chip. This system fits different Agilent mass spectrometers such as quadrupole time-of-flight (QTOF), single quad, triple quad and ion trap [3].

In this paper, recently developed Polaris-HR-chip-3C18 column was compared with previously released proteomic LC-chip columns (Protein ID-150 and Large Capacity chips, both packed with Zorbax SB-C18 stationary phase, Agilent Technologies, Santa Clara, CA, USA) and with a classical LC column. The purpose was to identify a maximum number of proteins in a complex matrix for future protein network studies.

Cardiovascular disease application was chosen for testing various columns. Integrative genomics and proteomics are indeed crucial in the continuous understanding of atherosclerosis and cardiovascular diseases [8,9]. Early detection of patients at risk is indeed vital and proteomics might bring interesting biomarkers for their identification. In the present report, we show the interest of new technologies and especially the Polaris-HR-chip-3C18 column to identify a maximum number of proteins present in atheroma plaques. That will help future works on the protein network understanding of atheroma plaque development and evolution.

2. Experimental

2.1. Reagents

Ammonium bicarbonate, formic acid (FA), Na_2HPO_4, methanol and acetonitrile (LC-MS grade), butylated hydroxyl toluene (BHT), dithiothreitol, iodoacetamide, protease inhibition mixture, 2,2,2-trifluoroethanol and TPCK-treated trypsin (from bovine pancreas) were from Sigma–Aldrich (St Louis, MO, USA). Water was purified using a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of protein extracts from atheroma plaques

Atheroma plaques were removed by endarterectomy from carotid arteries of three patients (total amount of plaque ≈ 5.5 g) suffering of carotid artery disease with atheroma deposit. This study conforms to the Declaration of Helsinki and its protocol was approved by the Ethics Committee of the ISPPC ("Intercommunale de Santé Publique du Pays de Charleroi") Hospital. Finally, all subjects gave their written informed consent. Protein extracts were prepared based on protocols previously described [10,11]. Briefly, plaques from patients were pooled, frozen with liquid nitrogen and directly crushed. Half of the powder (≈2.5 g) were placed in a 15 mL centrifuge tube and proteins were extracted with 4.0 mL of extraction buffer (0.15 M NaCl, 100 μM diethylenetriaminepentaacetic acid, 100 μM BHT, protease inhibitor mixture and 10 mM sodium phosphate, pH 7.4) with gentle shaking for 24 h at 4 °C. Extracted proteins (500 μL) were delipidated twice by diethyl-ether/methanol/water (7/3/1, v/v) solution. Proteins pellets were then unfolded (2,2,2-trifluoroethanol), reduced (dithiothreitol), alkylated (iodoacetamide) and trypsin digested [12]. Peptides were dissolved in 100 μL of FA (0.1% [v/v] in water). Samples were prepared in triplicate from the protein delipidation step.

2.3. LC-MS/MS process, data acquisition and analysis

Ten μL of the samples were injected into the LC-system and analyzed as described [12]. For classical LC method, analyses were performed using a rapid resolution LC system (RRLC 1200 series, (Agilent Technologies, Santa Clara, CA, USA). Peptides were separated on a Poroshell 120 EC-C18 column (100 × 2.1 mm I.D., 2.7 μm particle size) using a 105 min gradient of FA and acetonitrile [13]. The latter is the same as described in Table 1 with following flow rates: beginning at 0.2 mL/min during 5 min then increased linearly to 0.3 ml/min in 10 min and finally to 0.5 ml/min until end. An electrospray ionization source fitted on a QTOF 6520 series MS was used for the MS/MS analyses (Agilent Technologies, Santa Clara, CA, USA). Auto-MS/MS spectra were acquired in positive and high-resolution acquisition mode (4 GHz) [13]. For nano analysis, peptides were separated on an Agilent Technologies nanoLC Chip Cube II system, coupled to the QTOF, using a ProtID-150, a Large Capacity or a Polaris-HR nano-chip column. Technical details of the chips are

Table 1
Characterizations of Prot ID-Chip-150, Large Capacity chip and Polaris HR-Chip-3C18 columns and gradient details.

CHIP characteristics	ProtID-Chip-150	Large Capacity Chip	Polaris HR-Chip-3C18
Chemistry	Zorbax SB-C18	Zorbax SB-C18	Polaris C18-A
Particle size	5 μm	5 μm	3 μm
Porosity	300 Å	300 Å	180 Å
Enrichment column	40 nL	160 nL	360 nL
Analytical column dimensions	150 × 75 μm	150 × 75 μm	150 × 75 μm
Maximal Pressure	200 Bars (150)	200 Bars (150)	250 Bars (200)
Nano Flow (nL/min)	600	400	400
Sample Loading (μL/min at 3% B)	4.0	2.0	2.0
Gradient	Time (min)	% B	
	0	1	
	5	1	
Solvent A:	15	5	
FA 0.1% in water [v/v]	25	5	
	80	20	
Solvent B:	83	20	
90/10–Acetonitrile/FA 0.1% in water [v/v]	93	90	
	95	90	
	99	1	
	101	1	
	105	1	