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Visualization procedures for proteins and peptides on flat-bed monoliths and their effects on matrix-assisted laser-desorption/ionization time-of-flight mass spectrometric detection

Bert Wouters[a], Dominique J.D. Vanhoutte[b], Petra Aarnoutse[b], Adriaan Visser[b], Catherine Stassen[a], Bart Devreese[c], Wim Th. Kok[b], Peter J. Schoenmakers[b], Sebastiaan Eeltink[a,*]

[a] Vrije Universiteit Brussel, Department of Chemical Engineering, Pleinlaan 2, B-1050 Brussels, Belgium
[b] Van't Hoff Institute for Molecular Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
[c] Universiteit Gent, Department of Biochemistry and Microbiology, K.L. Ledeganckstraat 35, B-9000, Ghent, Belgium

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ABSTRACT

The present study concerns the application of visualization methods, *i.e.* coomassie-brilliant-blue-R staining (CBB-R), silver-nitrate staining, and fluorescamine labeling, and subsequent MALDI-MS analysis of intact proteins and peptides on the surface of flat-bed monoliths, intended for spatial two-dimensional chromatographic separations. The use of 100-μm thick macroporous poly(butyl methacrylate-*co*-ethylene dimethacrylate) flat-bed monoliths renders a fixation step obsolete, so that CBB-R and silver-nitrate staining and destaining could be achieved in 10–15 min as opposed to up to 24 h, as is typical on 2D-PAGE gels. The detection limits remained comparable. The compatibility of the monolithic layer with subsequent MALDI-MS analysis of individual proteins and peptide spots was investigated with regards to mass accuracy, mass precision, resolution, and signal intensity. When comparing results from MALDI-MS analysis of proteins and peptides on a flat-bed monolith to results obtained directly on stainless-steel target plates, significant losses in mass precision, signal intensity, and an increased variation in resolution were observed. In addition, a loss in signal intensity up to two orders of magnitude was observed when using monolithic layers. After CCB-R and silver-nitrate staining and destaining to disrupt the protein–dye complexes no MALDI spectra with significant S/N ratios could be achieved. After fluorescamine labeling heterogeneous signals were observed, which resulted from a distribution in the number of fluorescence-labeled lysine groups and from the presence of labeled derivatives that had undergone condensation reactions.

1. Introduction

The diversification of the cellular proteome is a result of protein synthesis by translation of mRNA and of the formation of protein isoforms by co- and post-translational modifications, such as oxidation, glycosylation, and acetylation [1]. This results in the formation of a highly complex mixture of proteins that differs between cell types and of which the composition and concentrations may fluctuate in time. Liquid chromatography–mass spectrometry (LC–MS) has become an indispensable tool for measuring changes in proteomic profiles, reflecting the biochemical condition of cells and tissues, which may result in the identification of biomarkers [1]. In 1D-LC–MS/MS experiments Kocher et al. demonstrated that the number of identified peptides was directly proportional to the peak capacity [2]. Multi-dimensional liquid-chromatography separations using a coupled-column approach (LC × LC) provide peak capacities (and peak-production rates, *viz.*, peak capacities per unit time) that are significantly higher than what can be achieved with one-dimensional (1D-) LC, resulting in even higher identification scores [3]. However, a disadvantage of LC × LC using a coupled-columns approach is the relatively long analysis time due to the sequential analysis of all first-dimension fractions.

In spatial chromatography, components are separated in the space domain with each peak being characterized by its coordinates in a plane or even a three-dimensional separation body. After completing the first separation, all "first-dimension fractions"

* Corresponding author. Tel.: +32 02 629 3324; fax: +32 02 629 3248.
E-mail address: seeltink@vub.ac.be (S. Eeltink).

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are analyzed in a single second-dimension analysis, which greatly enhances the productivity. A well-known example of a spatial two-dimensional separation is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [4]. 2D-PAGE involves the separation of proteins based on differences in their iso-electric point (p*I*) using iso-electric focusing, followed by a second-dimension size-based separation in a polyacrylamide gel. Proteins are usually visualized – and subsequently quantified – through a colorimetric reaction, such as silver-nitrate staining [5], coomassie-brilliant-blue staining [6], or *via* fluorescent labeling [7]. Protein spots of interest are excised from the gel, enzymatically digested, purified, and identified by elucidating the sequence coverage of the peptides using LC–MS^n. The main disadvantages of 2D-PAGE followed by LC–MS^n are that it is very labor intensive and that the total analysis time may amount to a week, due to the slow in-gel digestion step and the sequential processing of all protein spots selected for subsequent LC–MS^n analysis [8,9].

The use of monolith technology may help to overcome two other bottlenecks of 2D-PAGE, *i.e.* the poor repeatability between gels and the interfacing with mass-spectrometric detection. A prototype low-pressure instrument for spatial comprehensive capillary electrophoresis × reversed-phase LC (CE × RPLC) separation has been discussed in [10]. Flow control was realized by applying an electro-driven and/or a pressurized flow over the *X*- and *Y* direction, respectively. The second-dimension "column" in the prototype device included a flat-bed polymer-based monolithic separation layer with reversed-phase properties. An open channel was created for the first-dimension electrophoretic separation by etching away a copper wire with nitric acid after creating the monolith.

The feasibility of using a porous-polymer monolith for (matrix-free) surface-enhanced laser desorption/ionization time-of-flight mass spectrometry of small molecules (drugs and explosives) was first demonstrated by Peterson et al. [11]. Porous methacrylate monolithic features were prepared at pre-selected locations on stainless-steel target plates using a photo-initiated polymerization reaction and a mask featuring circular spots with a diameter of 3 mm. The mass spectra were virtually identical during hundreds of measurements, demonstrating the stability of the monolith. In a subsequent publication Svec' group demonstrated the use of monolithic porous layers for the separation of fluorescent-labeled proteins and peptides by thin-layer chromatography (TLC) [12]. To promote ionization, a matrix solution was applied on top of the spots prior to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) measurements. Svec' group also showed the feasibility of using desorption electrospray ionization (DESI) MS for the detection of peptides on the surface of a monolithic flat bed for thin-layer chromatography [13].

In the present study, we report on the preparation of 100-μm thick monolithic layers on glass slides and stainless-steel MALDI target plates. The flat-bed monoliths have the potential to be employed as stationary phases for spatial two-dimensional chromatography, and replace the cumbersome 2D-PAGE technology. Visualization procedures for proteins are invariably applied after 2D-PAGE analysis. These include coomassie-brilliant-blue-R staining, silver-nitrate staining, and fluorescent labeling. These staining methods were optimized for application with porous flat-bed monoliths. The potential of MALDI-MS detection of peptides and intact proteins from flat-bed monoliths and the effect of visualization procedures on subsequent analysis by MALDI-MS is discussed.

2. Experimental

2.1. Chemicals and reagents

Butyl methacrylate (BMA, 99%), ethylene glycol dimethacrylate (EDMA, 98%), 1,4-butanediol (99%), 1-propanol (99.9%), 2,2-dimethoxy-2-phenylacetophenone (DMPAP, 99%), cytochrome *c* from equine heart, boric acid (≥99.5%), ammonium bicarbonate (≥99%), trifluoroacetic acid (TFA, 99%), fluorescamine (99%), coomassie-brilliant-blue-R staining solution (CBB-R), angiotensin II, a "ProteoMass Peptide & Protein MALDI-MS Calibration Kit" containing $P_{14}R$ synthetic peptide (MH^+ 1533.86), and a ProteoSilver Plus silver-stain kit were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). α-Cyano-4-hydroxycinnamic acid (α-CHCA, 97%) was purchased from Acros (Geel, Belgium). Acetonitrile (HPLC Supra-gradient), acetone (analytical grade), and methanol (LC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Acetic acid was obtained from Merck (Darmstadt, Germany). A commercial peptide mixture (containing bradykinin (MH^+ 904.46), angiotensin I (MH^+ 1296.68), glu-fib (MH^+ 1570.677), ACTH (1–17) (MH^+ 2093.087), ACTH (18–39) (MH^+ 2465.199), and ACTH (7–38) (MH^+ 3657.93) with respective concentrations of 1; 2; 1.3; 2; 1.5 and 3 pmol/μL) was obtained from AB SCIEX (Darmstadt, Germany). A commercial protein mixture (containing ACTH (18–39) (MH^+ 2466.72), insulin (MH^+ 5734.59), ubiquitine (MH^+ 8565.88), cytochrome *c* (MH^+ 12361.50), and myoglobin (MH^+ 19652.56) with respective concentrations of 0.5; 2; 2; 5 and 10 pmol/μL) was obtained from LaserBiolabs (Sophia Antipolis, France). Milli-Q water (18.2 MΩ cm) was produced by an Arium 611UV Ultrapure Water System (Sartorius Stedim Biotech, Aubagne Cedex, France).

BMA and EDMA were purified by passing the liquids over activated basic alumina columns to purify the monomers (Sigma–Aldrich). Other solvents were used as received. Microscope glass slides (76 mm × 26 mm, 1 mm thick) were purchased from Menzel-Gläser (Braunschweig, Germany) and Teflon film (100-μm thick) was purchased from FluorTech BV (Heerhugowaard, The Netherlands).

For silver staining, the following solutions were prepared: a sensitizer solution was prepared from 35 volume parts methanol, 62 parts water and 3 parts ProteoSilver-Sensitizer solution. A silver solution was prepared from 35 volume parts methanol, 62 parts water and 3 parts ProteoSilver-Silver solution. A developer solution was prepared from 35 volume parts methanol, 59.9 parts water, 5 parts ProteoSilver-developer-1 solution, and 0.1 part ProteoSilver-developer-2 solution. Finally, a destainer solution was prepared from 35 volume parts methanol, 49 parts water, 8 parts ProteoSilver destainer A, and 8 parts ProteoSilver destainer B solution.

2.2. Instrumentation

An XLE-1000A/F microprocessor-controlled UV-Crosslinker (Spectroline, Distrilab, Leusden, The Netherlands) was used for the preparation of the polymer flat-bed monoliths. The polymerization reaction was initiated at 365 nm with an irradiation intensity of 30 μW/mm^2. The morphology and homogeneity of the flat-bed monoliths were evaluated based on micrographic images taken by field-emission scanning electron microscopy (SEM 525, Philips, Eindhoven, The Netherlands). Prior to SEM-experiments, a 2-nm gold coating was sputtered on the surface of the monolith to ensure electrical conductivity.

MALDI time-of-flight MS (MALDI-TOF-MS) measurements were carried out on a 4800+ MALDI TOF/TOF Proteomics Analyzer (AB SCIEX, Darmstadt, Germany). After spotting 1 μL of the peptide or protein mixture on a stainless-steel MALDI target plate or a stainless-steel MALDI target plate covered with a flat-bed monolith, 1 μL matrix solution (5 mg/mL α-hydroxycinnaminic acid dissolved in 60% ACN, 10 mM ammonium citrate, 0.1% TFA) was added. MS spectra were obtained in reflectron and linear mode for peptide and protein analysis, respectively, after acquiring 400 laser shots with a 200 Hz laser (Nd:YAG laser; 355 nm). The laser intensity was set at 5000 and 4000, respectively, for linear and reflectron