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Hydrophobin-coated plates as matrix-assisted laser desorption/ionization sample support for peptide/protein analysis

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ABSTRACT

Fungal hydrophobins are amphipathic self-assembling proteins. Vmh2 hydrophobin, prepared from mycelial cultures of the basidiomycete fungus *Pleurotus ostreatus,* spontaneously forms a stable and homogeneous layer on solid surfaces and is able to strongly absorb proteins even in their active forms. In this work, we have exploited the Vmh2 self-assembled layer as a novel coating of a matrix-assisted laser desorption/ionization (MALDI) steel sample-loading plate. Mixtures of standard proteins, as well as tryptic peptides, in the nanomolar–femtomolar range were analyzed in the presence of salts and denaturants. As evidence on a real complex sample, crude human serum was also analyzed and spectra over a wide mass range were acquired. A comparison of this novel coating method with both standard desalting techniques and recently reported on-plate desalting methods was also performed. The results demonstrate that Vmh2 coating of MALDI plates allows for a very simple and effective desalting method suitable for development of lab-on-a-plate platforms focused on proteomic applications.

Hydrophobins are a family of small amphipathic proteins (comprising ~100 amino acid residues) produced by filamentous fungi. They are able to self-assemble on hydrophobic or hydrophilic solid surfaces into thin amphiphilic layers, changing the hydropathy character of the surfaces [1]. Based on the structural features and properties of their aggregates, hydrophobins have been classified into two groups: class I and class II. Layers formed by class II hydrophobins are soluble in ethanol or sodium dodecyl sulfate (SDS)[1] aqueous solutions, whereas class I hydrophobins form highly insoluble membranes that can be solubilized only by strong acids such as trifluoroacetic acid (TFA) [2]. Various biotechnological applications have been reported by exploiting the interesting properties of these fungal proteins [3–5]. In particular, hydrophobin coating has been used as an intermediate layer to attach cells, proteins, or other biomolecules to surfaces for development of several biotools such as biosensors and DNA/protein microarrays [6–9].

A class I hydrophobin secreted by the basidiomycete fungus *Pleurotus ostreatus* has been purified and identified as Vmh2 [10]. Vmh2 behavior has been widely investigated both in solution [11] and in assembled form [12–14]. We have demonstrated that Vmh2 is spontaneously able to form a stable and homogeneous layer on hydrophilic or hydrophobic surfaces, changing their wettability [12]. The stability of the self-assembled coating has also been investigated in order to protect nanocrystalline material, such as porous silicon, used in optical devices for biosensing [13]. Furthermore, we have shown that Vmh2-coated surfaces are able to strongly bind proteins, including enzymes in their active form [14].

Following these results, Vmh2 hydrophobin layers have been tested in coating steel matrix-assisted laser desorption/ionization (MALDI) sample plates with the aim to develop a lab-on-a-plate platform focused on proteomic applications [15].

Ion suppression phenomena, due to salts, denaturants, and/or other reagents, in peptide/protein samples result in a significant decrease of signal sensitivity in MALDI analysis [16,17]. Most of the common desalting methods (e.g., high-performance liquid chromatography [HPLC], ZipTip, PD10) require relatively large sample volumes and/or are associated with sample loss [18]. These drawbacks can limit the advantages of MALDI–MS (mass spectrometry) in terms of sensitivity. To overcome these limits, several authors have suggested the use of *on-plate* sample treatment by

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[1] *Abbreviations used:* SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; AMBIC, NH_4HCO_3; TOF, time-of-flight; CHCA, α-cyano-4 hydroxycinnamic acid; S/N, signal-to-noise; DHB, 2,5-dihydroxybenzoic acid; SEM, scanning electron microscopy; S/N, signal/noise.

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properly treating the MALDI plate surface [15,19]. It is worth recalling that surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, introduced by Hutchens and Yip in 1993 [20,21], was the first approach to exploit on-plate sample desalting/enrichment. A number of in-house methods to cover MALDI plates with hydrophobic layers aimed at on-plate desalting/enrichment of samples have been further suggested [22–25].

In this article, we describe the surface treatment of MALDI sample plates with hydrophobin Vmh2, resulting in a simple and effective desalting method prior to MALDI analysis. To the best of our knowledge, this is the first report on the use of a biomaterial to improve the efficiency of MALDI–MS analysis of peptides/proteins. A comparative evaluation of the described technique with respect to other off-plate and on-plate desalting techniques—ZipTip microcolumns and paraffin-coated plates—has been made showing specific benefits of Vmh2 as a coating material of MALDI sample plates.

Materials and methods

Materials

Lysozyme from chicken egg white, ovalbumin from chicken egg white, myoglobin from equine heart, ribonuclease from bovine pancreas, α-lactalbumin from bovine milk, trypsin from bovine pancreas, and paraffin wax were purchased from Sigma (St. Louis, MO, USA). *P. ostreatus* mycelia (type: Florida no. MYA-2306) were purchased from American Type Culture Collection (ATCC; Italian distributor: LGC Standards, Sesto San Giovanni, Italy). PD-10 desalting columns were purchased from GE Healthcare (Milan, Italy). $ZipTip_{C18}$ microcolumns were purchased from Millipore (Vimodrone Italy). Calibration mixtures were purchased from AB SCIEX (Framingham, MA, USA).

Tryptic digests

Lysozyme and ovalbumin were dissolved at 5 μM in 0.5 M Tris–HCl (pH 8.0) containing 6 M guanidinium chloride and then reduced in 10 mM dithiothreitol for 2 h at 37 °C and carboxamidomethylated in 50 mM iodoacetamide for 30 min, in the dark, at room temperature.

Samples were desalted on a PD10 column using 10 mM NH_4HCO_3 (AMBIC) as eluting buffer. Fractions containing proteins were pooled, concentrated, and treated with trypsin (substrate/enzyme ratio, 50:1, w/w) for 18 h at 37 °C. Samples were lyophilized and dissolved in four different solutions—namely 0.1% formic acid, 10 mM AMBIC, 1 M NaCl, and 2 M urea—to test the effectiveness of on-plate washing of hydrophobin-coated plates.

$ZipTip_{C18}$ desalting

Desalting of peptide samples was performed by using $ZipTip_{C18}$ microcolumns according to manufacturer's instructions. Briefly, 0.1% TFA was added to samples to get pH < 4.0. The $ZipTip_{C18}$ was wetted with 10 μl of 50% acetonitrile and 0.1% TFA (three times) and equilibrated with 10 μl of 0.1% TFA (three times). Samples were slowly aspirated and dispensed 10 times, and then desalting was achieved by aspirating and dispensing 10 μl of 0.1% TFA (5 times). Finally, peptides were eluted with 10 μl of 50% acetonitrile and 0.1% TFA.

Standard proteins preparation

Standard proteins (lysozyme, myoglobin, ribonuclease, and α-lactalbumin) were dissolved at 0.1 mg ml^{-1} in 0.1 M sodium acetate (pH 3.6), 0.1 M sodium acetate (pH 5.8), 0.05 M sodium bicarbonate (pH 7.5), or 0.05 M sodium bicarbonate (pH 9.6).

Serum proteins preparation

Human serum, obtained from blood of a healthy donor and prepared by following the standard laboratory procedures, was provided by the blood bank center of the "Federico II" university hospital and used without any further treatment and/or purification.

Vmh2 purification

Vmh2 was prepared from *P. ostreatus* mycelia as described previously [10]. Preparation basically involves growing mycelia at 28 °C in static cultures in 2-L flasks containing 500 ml of potato dextrose (24 g/L) broth with 0.5% yeast extract. After 10 days of fungal growth, hydrophobins released into the medium were aggregated by air bubbling using a Waring blender and collected by centrifugation at 4000*g*. The precipitate was freeze-dried, left to dissolve under mechanical agitation with TFA for 2 h, and treated in a bath sonicator (operating at 35 kHz and 240 W) for 30 min. The TFA solution was dried in a stream of air, and Vmh2 was dissolved in 60% ethanol, resulting in a stock solution (100 μg ml^{-1}) that was further used to coat MALDI plates.

MALDI plate coating

Vmh2 solution (1 μl) was spotted on each MALDI plate well. After 1 h, samples were dried for 10 min at 80 °C and washed with 60% ethanol. Deposition was repeated twice, and finally the plate was washed with 2% SDS at 100 °C for 10 min. To obtain mass spectra, 1 μl of sample (tryptic peptides, standard proteins, or human serum) was spotted on MALDI plate wells coated by Vmh2. After 5 min of incubation at room temperature, wells were washed three times with water. Finally, the appropriate matrix was added and left to crystallize. Vmh2 coating can be removed by washing the MALDI plate with 10% TFA (by gently polishing the surface) followed by washing with acetonitrile, water, and acetone.

Other coating methods were tested in comparison with hydrophobin coating. Mineral oil, glycerol [25], and paraffin wax [26] were used according to the procedures described previously.

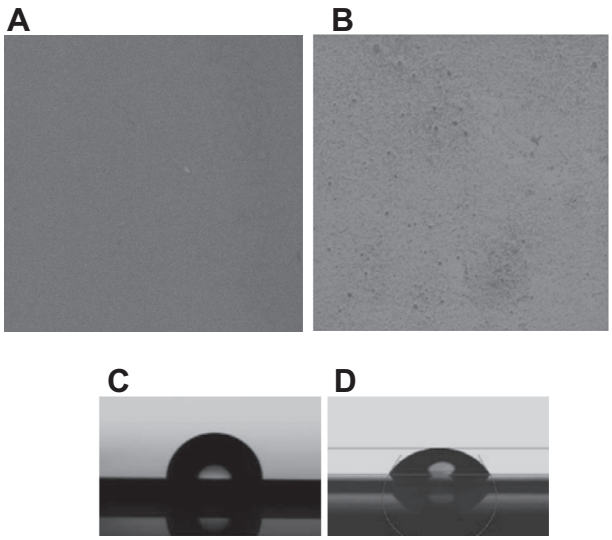

Fig.1. (A, B) SEM images of bare steel (A) and Vmh2-coated steel (B). (C, D) Water contact angle of a water drop on steel (C) and on Vmh2-coated steel (D).