International Journal of Mass Spectrometry 353 (2013) 54–59

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Fluorophore-assisted laser desorption/ionization-mass spectrometry (FALDI-MS)

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ARTICLE INFO

Article history:
Received 9 May 2013
Received in revised form 5 July 2013
Accepted 5 July 2013
Available online 15 July 2013

Keywords:
Fluorophore-assisted LDI-MS
Visible-wavelength MALDI-MS
Peptides
Fluorophores
Fluorescent labeling
Beta-amyloid peptides

ABSTRACT

The direct analysis of fluorescently labeled peptides by laser desorption/ionization-mass spectrometry (LDI-MS) is demonstrated for the first time. Peptides labeled on their N-termini with rhodamine B were analyzed by MS and tandem MS (MS/MS) using a recently developed visible-wavelength LDI-MS instrument. Labeling with a fluorescent dye leads to soft LDI of peptides in the absence of a matrix. The MS/MS spectra were simplified because only a and b rhodamine B-containing peptide fragment ions were observed, which allowed for easy spectral interpretation and *de novo* sequencing of peptides. In mixtures, the rhodamine B labeled peptides demonstrated a greater ionization efficiency, and therefore selectivity, compared to ionization of the unlabeled peptides. Additionally, FALDI-MS was used to directly analyze beta-amyloid peptides that were N-terminally labeled with the fluorophore HiLyte Fluor™ 555. The described FALDI-MS methodology provides a novel way to ionize and structurally characterize specifically labeled peptides.

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1. Introduction

Laser desorption/ionization mass spectrometry (LDI-MS) is a technique that has been used to analyze the samples such as organic molecules and biomolecules [1,2]. This technique can be used with lasers that range in wavelengths from the ultraviolet (UV) to the infrared (IR) region of the electromagnetic spectrum, but it is most commonly performed with UV lasers on time-of-flight (TOF) mass spectrometers. With the addition of a matrix that absorbs the light and helps with desorption and ionization of the analyte, more types of samples with increasing molecular weights have been analyzed efficiently by matrix-assisted LDI (MALDI)-MS [3–6].

It is an essential requirement of a good MALDI-MS matrix to efficiently absorb laser light impacting the sample and transfer energy to the analyte. Many dyes and fluorophores have high absorption coefficients, and have been tested as matrices for MALDI-MS [7–15]. Such studies have been done mostly with visible-wavelength MALDI-MS [7–14]. Fluorescent dyes have also been used as matrix additives to improve the ion intensities recorded by UV MALDI-MS [15]. Additionally, Pashkova et al. have investigated fluorescent dye labeling as a way to improve ionization efficiency of peptides in the presence of a UV MALDI matrix [16] and to identify proteins by HPLC–MALDI-MS [17]. Most of the previous studies have either used free fluorophores as matrices or analyzed fluorescently labeled molecules by MALDI-MS.

Here, we apply an AP visible-wavelength LDI source that is coupled to an ion trap (IT) mass spectrometer to analyze fluorescently labeled peptides in the absence of a matrix. A fluorophore was covalently bonded to the N-terminus of the peptide to absorb visible-wavelength light from a frequency-doubled Nd:YAG laser (emits 532 nm), which allowed for the detection of the peptides by LDI-MS (Scheme S1). The peptide ions formed were successfully analyzed without the addition of a visible-wavelength matrix by LDI MS and MS/MS. Since we have demonstrated that a covalently attached fluorophore facilitates desorption and ionization, we name this methodology fluorophore-assisted LDI-MS (FALDI-MS).

2. Experimental

Acetonitrile (CH_3CN, HPLC grade), trifluoroacetic acid (TFA, purity >98%), caffeine, ultramark 1621, and water (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). The indium tin oxide (ITO)-coated glass slides were purchased from Bruker Daltonics (Bremen, Germany). Peptide MRFA (Met-Arg-Phe-Ala), 2-amino-3-nitrophenol, sinapinic acid, poly(propylene glycol) average $M_n \sim 2000$ Da, and poly(propylene glycol) average $M_n \sim 2700$ Da were purchased from Sigma (St. Louis, MO).

Two different rhodamine B labeled peptides (purity > 95%) were obtained from Ohio Peptide (Powell, OH). The amino acid

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http://dx.doi.org/10.1016/j.ijms.2013.07.004

sequences of labeled peptides were rhodamine B-GILGFVFTL-COOH and rhodamine B-TLLYVLFEV-COOH. The peptides were synthesized using a standard solid-phase synthesis procedure, labeled with rhodamine B and purified by HPLC by the manufacturer. Unlabeled GILGFVFTL peptide was also purchased from Ohio Peptide. Two beta-amyloid peptides labeled with HiLyte Fluor™ 555 (purity > 95%) were obtained from AnaSpec (Fremont, CA). The sequences of these peptides were HiLyte Fluor™ 555-DAEFRHDSG and HiLyte Fluor™ 555-DAEFRHDSGYEVHHQK. All these samples were analyzed without further purification.

Peptide samples were dissolved at a concentration of 10 mg/ml in 1:1 (v:v) H_2O:CH_3CN solution containing 0.1% TFA since all samples were analyzed in positive ion mode. Sample spots for FALDI-MS were prepared by depositing 3 μl of a sample on an ITO-coated glass slide. For visible-wavelength MALDI-MS experiments, the matrix 2-amino-3-nitrophenol was dissolved in acetone containing 0.1% TFA at a concentration of 10 mg/ml. Sample spots being analyzed were prepared by mixing 3 μl of a sample with 3 μl of the matrix. Using the matrix-to-analyte mass concentration ratio of 1:1 gave the best results for visible-wavelength MALDI-MS analyses of peptides although other matrix-to-analyte ratios were studied as well [18].

The peptide mixtures were prepared by mixing 3 μl of the fluorescently labeled peptides with 3 μl of labeled or unlabeled peptides (angiotensin II, a tryptic digest of myoglobin, and a peptide mixture of angiotensin II, angiotensin I, substance P, and bombesin). Each fluorescently labeled peptide was mixed with unlabeled peptides of equal concentration (10 mg/ml) except in the case of myoglobin digest (1.1 mg/ml). For each mixture only a fluorescently labeled peptide and one other peptide sample were mixed together. All samples were spotted onto an ITO-coated glass slide and dried under vacuum in a desiccator.

AP visible-wavelength LDI-MS instrument used in this study was described in our recent report [18]. Briefly, the instrument was built by coupling a frequency-doubled Nd:YAG laser (Minilite I, Continuum, Santa Clara, CA), which emits approximately 4.8 ns pulses at a wavelength of 532 nm, to an electrospray ionization (ESI)-IT-MS instrument (LCQ Deca XP^{Plus}, Thermo Finnigan, San Jose, CA) in transmission geometry. The instrument was operated in positive ion mode using Xcalibur (Thermo Scientific) software. The temperature of the ion transfer capillary was set to 315 °C and the sample target voltage applied to the ITO-coated glass slide was 4.7 kV [18]. The IT-MS instrument was calibrated in the m/z range of 100–2000 using a standard mixture containing caffeine, the peptide MRFA, and the synthetic polymer Ultramark 1621. Additionally, the IT-MS was calibrated in the high mass range to measure ions with m/z values of up to 4000. A mixture of poly(propylene glycol) with an average mass of 2000 Da and poly(propylene glycol) with an average mass of 2700 Da was analyzed. ESI-MS was used to calibrate the instrument for both mass ranges.

UV LDI and MALDI-MS experiments under vacuum were performed using an UltrafleXtreme MALDI-MS instrument (Bruker Daltonics). This instrument uses a pulsed Smartbeam II laser, which emits light at a wavelength of 355 nm, in reflection geometry. For LDI-MS experiments, 1 μl of each peptide sample was spotted onto a MTP 384 ground steel target plate (Bruker Daltonics) after the instrument was calibrated using a peptide calibration standard mixture. For UV MALDI-MS, 1 μl of sinapinic acid matrix (c = 10 mg/ml in 1:1 (v:v) H_2O:CH_3CN) was mixed with 1 μl of peptide solution (c = 10 mg/ml) and spots were dried under vacuum in a dessicator. The mass spectra were acquired using FlexControl (Bruker Daltonics) software.

MS-Product and MS-Digest features of Protein Prospector software, which can be found at http://prospector.ucsf.edu/, were used to determine the fragmentation pattern of the analyzed peptides and protein digest samples.

3. Results and discussion

The concept of FALDI-MS is shown in Scheme 1. A fluorescent dye (*e.g.*, rhodamine B) is covalently bound to the peptide's N-terminus through a labeling procedure. The laser light is absorbed by the fluorophore leading to desorption and ionization of the fluorescently labeled peptide, which is further analyzed by the mass spectrometer.

Initially, two rhodamine B-labeled peptides were analyzed by visible-wavelength FALDI-MS. The first peptide analyzed had the sequence rhodamine B-GILGFVFTL-COOH. Fig. 1a shows FALDI-MS spectrum of this peptide, which is dominated by the protonated peptide ion (m/z = 1391.27). A sodiated peptide ion was observed in much lower intensity compared to the base peak. Fig. 1b shows FALDI-MS/MS spectrum of the peptide. The MS/MS spectrum of the protonated peptide ion shows formation of a and b ions as well as the loss of water from the parent ion and from the b_8 ion. It is important to note that all a and b ions contain rhodamine B cation (m/z 443 upon loss of chloride anion), which was covalently bound to the peptide upon the loss of water.

The second peptide analyzed was rhodamine B-TLLYVLFEV-COOH. The FALDI-MS spectrum of the peptide (Fig. 1c) is dominated by the protonated peptide ion (m/z = 1522.00). An additional peak (m/z = 1420.47) was observed, but with lower intensity compared to the base peak. This truncated peptide has the sequence rhodamine B-LLYVLFEV-COOH and its m/z value shows that a threonine residue (mass difference 101) is missing from the N-terminus of the complete fluorescently labeled peptide. Hence, this peak results from incomplete synthesis of the fluorescent peptide, which was confirmed by the UV MALDI mass spectrum obtained from the peptide's manufacturer. Fig. 1d represents the MS/MS spectrum of protonated rhodamine B-TLLYVLFEV-COOH ion, which also shows formation of rhodamine B-containing a and b ions.

The a and b fragment ions that appear in the MS/MS spectra of both fluorescent peptides can be used readily to determine their amino acid sequences. Fig. 1b and d shows the sequences of rhodamine B-labeled peptides that were determined based on the differences between consecutive b ions (a ions can also be used for sequence determination). The mass difference between two adjacent peptide fragments of the same type corresponds to the loss of one amino acid residue. For example, the difference between b_8 and b_7 fragments in Fig. 1b corresponds to the mass of a threonine residue. This analysis can be done for each adjacent fragment peak to determine the sequence of the entire peptide, and it can be an efficient procedure for peptide *de novo* sequencing. The peptide sequences obtained matched those supplied by the peptide manufacturer.

The sequencing of labeled peptides was straight forward because FALDI MS/MS spectra of these peptides are less complicated than the corresponding MALDI MS/MS spectra of the unlabeled peptides. This was confirmed by comparison of the FALDI-MS/MS spectrum of the protonated rhodamine B-GILGFVFTL peptide ion (Fig. 1b) to the spectra of unlabeled protonated peptide ion (m/z ~ 966.6, Fig. 2a and c) obtained using AP visible-wavelength MALDI-MS/MS (Fig. 2b) and UV MALDI-MS/MS (Fig. 2d). Clearly, the MS/MS spectra of fluorescently labeled peptides are less complicated (*i.e.*, contained less types of fragment ions) than the MS/MS spectra of unlabeled peptides. Additionally, FALDI MS/MS avoids the inability of an ion trap mass analyzer to detect fragments smaller than ~1/3 of the mass of a selected peptide ion. The addition of the rhodamine B fluorophore shifts fragment ion masses in the detectable mass range for the peptides that have m/z values up to ~1500.

The fluorescently labeled peptides were further mixed with the matrix 2-amino-3-nitrophenol and analyzed by AP visible-wavelength MALDI-MS to check if they would ionize similarly

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