The Analyses of Fluorescently Labeled Biomolecules Using Ultraviolet-Wavelength Fluorophore-Assisted Laser Desorption/Ionization-Mass Spectrometry (UV FALDI-MS)

Raymond E. West III, Justin B. Jacobs, and Dragan Isailovic*

Department of Chemistry and Biochemistry, University of Toledo, Toledo, OH 43606

Abstract

It was demonstrated recently that peptides labeled with fluorescent dyes on the N-terminus can be softly ionized using a visible-wavelength laser, and analyzed by fluorophore-assisted laser desorption/ionization-mass spectrometry (FALDI-MS) without the addition of a matrix [International Journal of Mass Spectrometry 353 (2013) 54-59]. In this study, UV FALDI-MS was used to analyze fluorescently labeled peptides and proteins as well as an oligosaccharide and a lipid sample that were fluorescently labeled. The results obtained demonstrate that FALDI-MS is applicable to various types of biomolecules and fluorophores, and can be performed using UV laser-based LDI-MS instruments. UV-FALDI MS/MS confirms that N-terminal labeling of peptides with dyes such as rhodamine B and carboxyfluorescein contributes to favorable formation of *a* and *b* ions upon fragmentation of fluorescently labeled peptides by laser-induced dissociation (LID). Moreover, UV FALDI-MS enables soft ionization of fluorescently labeled proteins such as insulin and cytochrome C in the absence of a matrix. Additionally, a protocol for efficient labeling of peptides and proteins with coumarin for subsequent FALDI and MALDI-MS analyses is presented.

Keywords: Fluorophore-assisted LDI-MS, UV MALDI-MS, Peptides, Proteins, Fluorophores, Fluorescent labeling

*Corresponding author: Dragan Isailovic, University of Toledo, Department of Chemistry and Biochemistry, MS 602, 2801 W. Bancroft St., Toledo, Ohio, 43606; Phone: 419-530-5523;

Fax: 419-530-4033. E-mail address: Dragan.Isailovic@utoledo.edu

Introduction

Laser desorption/ionization mass spectrometry (LDI-MS) has been successfully used to analyze samples such as peptides [1,2], polypeptides and proteins [2,3], polymers [4], and organic molecules [5]. It is typically performed with ultraviolet [1,2] or infrared lasers [3,4]. However, LDI-MS has a limited mass range and often leads to increased fragmentation of the analytes due to high laser pulse energies needed for ionization to occur [3]. This led to the development of softer ionization techniques to overcome the limitations of LDI-MS. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) overcame most of the limitations of LDI-MS. This technique utilizes a matrix that is able to absorb the laser light and facilitate desorption and soft ionization of the analytes [6-9]. Therefore, MALDI-MS is a softer ionization technique that can analyze much larger samples than LDI-MS, such as biopolymers [6-9].

LDI and MALDI-MS have been used to analyze fluorescent molecules. Since both highly fluorescent and low fluorescent dyes absorb light strongly, many of them have been studied as potential matrices for visible-wavelength and UV MALDI-MS [10-17]. For example, coumarin 510, coumarin 519, and rhodamine isothiocyanate (RITC) have been tested as visible-wavelength MALDI matrices [10,11]. Fluorescent dyes have also been used as dye co-matrices for the analysis of protein samples by visible-wavelength MALDI-MS and the investigation of the mechanism of UV MALDI [18-20]. The fluorescent dyes, such as coumarin, can covalently label the ϵ -amine of lysine residues and the N-termini of peptides and proteins in order to investigate labeled biomolecules in fluorescence-based applications [21].

Fluorescently labeled peptides were analyzed by MALDI-MS to improve the ion yield and fragmentation. Coumarin tags were applied to enhance both MALDI-MS and tandem mass

spectrometry (MS/MS) signal of fluorescently labeled peptides [22,23]. The MALDI-MS analysis of a fluorescently labeled peptide in the presence of α -cyano-4-hydroxycinnamic acid (CHCA) matrix enabled higher sensitivity of postsource decay (PSD) fragments, and mostly *b*-series fragment ions were observed [24]. Similarly, improved peptide sequencing due to formation of *b* ion series was demonstrated by MALDI-MS analysis of a peptide labeled with dansyl chloride [25]. Additionally, MALDI-MS of fluorescently labeled glycopeptides has been shown to increase the ionization yield of these samples [26]. MALDI-MS was also used to determine the number of fluorescein isothiocyanate (FITC) and biotin labels bound to protein samples [27]. Although many studies that apply fluorophores for the labeling of biomolecules have focused on covalent bonding, several studies examined noncovalent dye-biomolecule complexes using MALDI and electrospray ionization (ESI)-MS [28-30].

While MALDI-MS analysis of fluorescently labeled peptides is beneficial, fluorescent biomolecules have been analyzed by LDI-MS in the absence of a matrix. Dipeptides containing natively fluorescent amino acids phenylalanine, tyrosine, and tryptophan were investigated by UV LDI-MS [1]. Several different glycosphingolipids were fluorescently labeled with nitrobenz-2-oxa-1, 3-diazole (NBD) and analyzed without the use of an external matrix by LDI-MS [31]. Additionally, these glycolipids were metabolically labeled with NBD in this study enabling direct, matrix-free UV LDI-MS analysis of these biomolecules [31].

Recently, we demonstrated that fluorescently labeled biomolecules can be analyzed by visible-wavelength LDI-MS in the absence of a MALDI matrix, and this technique was named fluorophore-assisted laser desorption/ionization (FALDI)-MS [32]. Fluorophores such as rhodamine B and HiLyte Fluor 555 were covalently bound to the N-termini of the peptides to enable selective and soft ionization of the labeled peptides in the absence of a MALDI matrix

[32]. The fluorophore absorbed energy from the visible-wavelength laser, which allowed for the soft desorption and ionization of the samples [32]. Here, we report that FALDI-MS can be performed using UV lasers (355 nm) to analyze not only peptides but also other types of biomolecules, such as proteins, a lipid, and an oligosaccharide that were labeled using UV-absorbing fluorescent dyes.

Experimental

Acetonitrile (CH₃CN, HPLC grade), trifluoroacetic acid (TFA, purity >98%), dimethyl sulfoxide (DMSO), and water (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). 7-methoxycoumarin-3-carboxylic acid, succinimidyl ester (coumarin) and 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3 β -ol (NBD-cholesterol) were purchased from Invitrogen (Carlsbad, CA) and Cayman (Ann Arbor, MI), respectively. The Microcon YM-3 centrifugal filter devices, CHCA, sinapinic acid, maltoheptaose, bradykinin acetate salt, insulin from bovine pancreas, and cytochrome C from equine heart were purchased from Sigma (St. Louis, MO). The vacuum concentrator used was from Eppendorf (Hamburg, Germany).

The rhodamine B labeled peptide with the sequence rhodamine B-GILGFVFTL-COOH (purity > 95%) was obtained from Ohio Peptide (Powell, OH). This peptide was synthesized using a standard solid-phase synthesis procedure, labeled with rhodamine B, and purified by HPLC by the manufacturer. Several HPLC-purified fluorescently labeled peptides (purity > 95%) were obtained from AnaSpec (Fremont, CA). These peptides include 5-carboxytetramethylrhodamine (TAMRA)-angiotensin II, carboxyfluorescein (FAM)-bombesin, and FAM-angiotensin II. The 2-aminobenzamide (2-AB) labeling kit for glycans was purchased from Prozyme (Hayward, CA).

UV FALDI and MALDI-MS experiments were performed under vacuum using an UltrafleXtreme MALDI TOF/TOF-MS instrument (Bruker Daltonics). This instrument utilizes a pulsed Smartbeam II laser that emits light at a wavelength of 355 nm. Fluorescently labeled peptide samples were dissolved at a concentration of 1 mg/mL in a 1:1 (v:v) H₂O:CH₃CN solution and all samples were analyzed in positive ion mode. Sample spots for UV FALDI-MS were prepared by depositing 2 µL of the sample onto a stainless steel MALDI plate (MTP 384 ground steel target plate, Bruker Daltonics). For UV MALDI-MS experiments, the matrices CHCA and sinapinic acid were dissolved in a 1:1 (v:v) H₂O:CH₃CN solution containing 0.1% TFA at a concentration of 10 mg/mL. The sample spots being analyzed were prepared by mixing 1 µL of the sample with 1 µL of the matrix onto a MTP 384 ground steel target plate. The sample spots were dried under vacuum using a desiccator. After the instrument was calibrated using a peptide calibration standard mixture, the mass spectra were acquired using FlexControl (Bruker Daltonics) software. Mass spectra of peptides, a lipid, and an oligosaccharide were acquired in reflectron TOF mode, while mass spectra of proteins were acquired in linear TOF mode. MS/MS analyses by laser-induced dissociation (LID) were performed using a LIFT TOF/TOF method on the UltrafleXtreme MALDI TOF/TOF-MS instrument [33]. The MS/MS spectra were collected for 500 laser shots at four different locations on a sample spot for a total of 2000 acquisitions with the laser energy setting being higher for fragmentation than for the precursor ion selection. Data analysis was carried out using the flexAnalysis (Bruker Daltonics) software.

The following procedure was used for the labeling of peptides with coumarin. The peptides were dissolved in 1:1 (v:v) H₂O:CH₃CN at a concentration of 10 mg/mL. A coumarin solution was prepared by dissolving coumarin in DMSO at a concentration of 1 mg/mL. 20 µL of the peptide sample was mixed with 4 µL of ammonium bicarbonate buffer (pH 8.3) and 20 µL of

coumarin. 20 μL aliquots of the coumarin dye were added every 30 minutes until a 3:1 (v:v) coumarin:peptide ratio was achieved. The solution was vortexed briefly and then incubated at room temperature in the dark after each addition of coumarin. The fluorescently labeled sample was diluted 100 times and then analyzed with UV MALDI-MS by spotting 1 μL of the labeled peptide with 1 μL of CHCA onto the stainless steel MALDI plate.

The procedure for labeling proteins with coumarin was very similar to the peptide labeling procedure used. The proteins were dissolved in 1:1 (v:v) $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ at a concentration of 1 mg/mL. A coumarin solution was prepared by dissolving coumarin in DMSO at a concentration of 1 mg/mL. The proteins were then fluorescently labeled with coumarin by adding 20 μL of coumarin to the solution consisting of 20 μL of the protein sample and 4 μL of ammonium bicarbonate buffer (pH 8.3). Coumarin dye was added in 20 μL increments every 30 minutes until a 4:1 (v:v) coumarin:protein ratio was achieved. The solution was vortexed briefly and then incubated at room temperature in the dark after each addition of coumarin. The fluorescently labeled sample was diluted 10 times and then analyzed with UV MALDI-MS by spotting 1 μL of the labeled protein with 1 μL of sinapinic acid onto the stainless steel MALDI plate.

The fluorescently labeled proteins were separated from the unbound coumarin fluorophore with a Microcon centrifugal filter device with a 3 kDa cutoff. This was performed using the procedure that is provided with the device. The fluorescently labeled samples were first diluted 100 times with 1:1 (v:v) $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ in order to dilute the amount of DMSO in the sample. The filter manual recommends < 5% DMSO to be placed in the filter devices. The diluted, unfiltered samples were placed into the filter devices and centrifuged at 14000g for 120 minutes. After centrifugation, the remaining sample was collected, excess solvent was

evaporated with a heated centrifuge, and then dissolved in 10 μL of 1:1 (v:v) $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ to preconcentrate the protein. This ensured that the fluorescently labeled protein sample was free of excess fluorophore before being analyzed by UV FALDI and MALDI-MS.

The limit of detection (LOD) of NBD-cholesterol by FALDI-MS was determined by finding the minimum concentration that yielded a signal-to-noise greater than three. The mass spectrum was acquired for a total of 2000 laser shots.

Results and Discussion

As shown in our previous study [32], FALDI-MS is a technique that uses a fluorescent dye (e.g., rhodamine B or HiLyte Fluor 555) that is covalently bound to the N-terminus of a peptide for soft ionization of peptides by LDI. The fluorophore absorbs the laser light, which leads to soft desorption and ionization of the fluorescently labeled peptide. The original FALDI-MS work used a frequency-doubled Nd:YAG laser (Minilite I, Continuum, Santa Clara, CA), which emits light at 532 nm in transmission geometry [32]. Here, FALDI-MS is demonstrated using a pulsed Smartbeam II laser of an UltrafleXtreme MALDI-MS instrument that emits light at a wavelength of 355 nm in reflection geometry.

Initially, UV FALDI-MS was used to analyze several peptides that were fluorescently labeled on the N-termini by different visible-wavelength absorbing dyes. It is important to note that FALDI-MS was successfully performed on samples that had relatively low absorbance at 355 nm without the addition of a matrix. The first sample that was analyzed was rhodamine B-GILGFVFTL-COOH. The visible-wavelength (532 nm) FALDI-MS spectrum showed soft ionization of the fluorescently labeled peptide [32]. Figure 1 shows the UV FALDI-MS spectrum of this rhodamine B labeled peptide. The base peak (m/z 1390.975) in the mass spectrum is the protonated ion of the fluorescently labeled peptide, and a less intense sodiated ion of the

fluorescently labeled peptide (m/z 1413.168) is also observed. Table S1 shows the in-solution values of absorption maxima and the extinction coefficients for each of the fluorophores used in this study. Rhodamine B has an absorption maximum at 543 nm (extinction coefficient 106,000 $\text{cm}^{-1}\text{M}^{-1}$), and a small local maximum at 356 nm (extinction coefficient 8300 $\text{cm}^{-1}\text{M}^{-1}$). Even though rhodamine B has relatively weak absorbance at 355 nm, UV FALDI-MS enables fairly soft analysis of the rhodamine B labeled peptide. There are a few low-intensity fragment peaks that were observed in the UV FALDI-MS spectrum of this peptide (Figure 1). The peaks in the low mass range could not be identified as sequence ions of the analyzed peptide. Some of the low-intensity fragments in the higher mass range are likely due to photodegradation of the rhodamine dye [34]. For example, the peak at m/z 1346.859 is due to the loss of CO_2 from rhodamine B labeled peptide. These fragments were not observed by visible-wavelength FALDI-MS [32]. However, the UV LDI-MS instrument used in this study acquires mass spectra with improved mass resolution, mass accuracy, and m/z range in comparison to the instrument we used for visible-wavelength FALDI-MS previously [32].

Peptides that were labeled with other fluorophores on the N-terminus were analyzed as well. The FALDI-MS spectrum of FAM labeled angiotensin II is shown in Figure 2a. The protonated ion (m/z 1404.615) is the base peak in the spectrum, and minimal fragmentation is observed in the low mass range of this spectrum. FAM has an absorption maximum at 495 nm (75,000 $\text{cm}^{-1}\text{M}^{-1}$) with baseline absorbance at 355 nm (Table S1). The FALDI-MS spectrum of FAM-bombesin is shown in Figure 2b. The mass spectrum of this sample was typical for a fluorescently labeled peptide, in which the base peak was the protonated ion (m/z 1995.914) with a less intense sodiated ion peak (m/z 2017.964). In addition, Figure S1 shows the FALDI-MS spectrum of TAMRA-angiotensin II. The protonated ion (m/z 1458.759) is the base peak in the

spectrum, with a couple fragment peaks observed. Therefore, FALDI-MS could be performed on TAMRA labeled peptides.

The N-terminally labeled peptide samples were also analyzed by FALDI-MS/MS to determine whether the same fragmentation patterns that were observed previously with visible-wavelength FALDI-collision-induced dissociation (CID)-MS/MS [32] occur with UV FALDI-LID-MS/MS. Only *a* and *b* rhodamine B-containing fragment ions were observed in the visible-wavelength FALDI-MS/MS spectra of rhodamine B labeled peptides, which made peptide sequencing simpler than using UV MALDI-MS/MS of unlabeled peptides [32]. Figure S2 shows the FALDI-MS/MS spectrum of the protonated ion (m/z 1390.975) of rhodamine B-GILGFVFTL-COOH. Only *a* and *b* fluorophore-containing fragment ions were identified in the MS/MS spectra, as observed previously for the same peptide with visible-wavelength FALDI-MS/MS [32]. Figure S3 shows the FALDI-MS/MS spectrum of the protonated ion (m/z 1404.615) of FAM-angiotensin II. The same type of fragmentation pattern was observed with only *a* and *b* fluorophore-containing fragment ions being identified. The precursor ions and fragments detected for all of the N-terminally labeled peptides were singly-charged ions.

The fluorophore used for the labeling of both peptides and protein samples was coumarin. Coumarin is another common fluorescent dye that labels the N-termini and the side chain of lysines in peptides and proteins, and it has an absorption maximum at 358 nm. Therefore, this is a very suitable fluorescent dye for UV FALDI-MS experiments. The coumarin dye that was used is a succinimidyl ester. The reactive ester of the coumarin dye will react with the N-terminus or the amine of a lysine residue of the peptide to form a stable amide bond through the loss of the succinimidyl group [35].

The coumarin labeling procedure for peptides was optimized first. A couple of different procedures for labeling peptides with coumarin dyes [10,22,23] were tested to find the one that works the best for the labeling of the peptide bradykinin. A protocol involved an one-time addition of coumarin to the peptide sample [10], while others add the coumarin in increments every thirty minutes [22,23]. In both cases the labeled peptide was observed in the spectrum; however, unlabeled peptide was detected as well [10,22]. The procedure was optimized by considering different factors that allowed for the complete labeling of the peptide sample with no unlabeled peptide being detected by UV MALDI-MS.

The complete labeling of both peptide and protein samples without any observable unlabeled sample peaks was achieved using the procedure described in the Experimental section. The solvents that were used during the labeling of peptides and proteins with coumarin played an important role and influenced the results obtained by FALDI and MALDI-MS. DMSO is not ideal for MALDI-MS sample preparation since it evaporates slowly, which leads to poor crystallization of the sample. However, DMSO allowed for much better labeling efficiency versus methanol or acetonitrile because coumarin is more soluble in DMSO than in the other solvents. The volumes of the peptide ($c = 10 \text{ mg/mL}$), buffer, and the dye ($c = 1 \text{ mg/mL}$) were not important but the initial ratio 5:1:5 (v:v:v) was found to be ideal. When there was less than a 3:1 (v:v) ratio of coumarin to peptide solution there was still detectable amounts of the unlabeled peptide, and at higher ratios of dye to peptide there was not any observable difference.

This coumarin labeling procedure was used to label bradykinin (RPPGFSPFR) on the N-terminus of the peptide. Figure 3 shows the MALDI-MS spectrum of coumarin labeled bradykinin. Only the protonated coumarin labeled peptide (m/z 1262.552) was observed, while no unlabeled peptide was detected at the calculated m/z of protonated bradykinin (1060.568).

Since there are no lysine residues present in bradykinin, it is expected to detect only the addition of one coumarin ($\Delta m/z$ 202) to the N-terminus of the peptide. MALDI-MS analysis of coumarin labeled bradykinin (Figure 3) demonstrated that our labeling procedure works well for peptides, and it was then applied for labeling of two proteins that contain multiple coumarin binding sites.

The proteins that were labeled were bovine insulin (2 N-termini, 1 lysine) and equine cytochrome C (acetylated N-terminus, 19 lysines). A similar labeling procedure was used as for peptide labeling with the exceptions of adding coumarin incrementally to achieve a 4:1 (v:v) coumarin:protein ratio for 120 minutes. Each sample was analyzed with UV MALDI-MS to ensure that the expected number of coumarin molecules was bound to the protein samples. After coumarin binding to protein was confirmed by MALDI-MS, labeled proteins were separated from any free coumarin in the sample using centrifugal filter devices with 3 kDa cutoff before being analyzed by UV FALDI-MS. Free fluorophore has the potential of behaving as a matrix; therefore, it had to be removed before FALDI-MS analysis.

The first protein that was analyzed was bovine insulin since it has relatively low molecular weight (MW ~5.7 kDa) and only three coumarin binding sites. After separation from free coumarin by a filter device, the labeled insulin was analyzed by UV MALDI-MS to ensure that the sample stayed intact during separation and that no free fluorophore was detected. In order to achieve better resolution and intensity of the coumarin labeled peaks, the sample was diluted 100 times. Figure 4a shows the MALDI-MS spectrum of coumarin labeled insulin with sinapinic acid as the matrix. The base peak in the spectrum corresponds to three coumarin molecules bound to insulin (m/z 6339.948), and less intense peaks correspond to two coumarin molecules (m/z 6137.926) and one coumarin molecule (m/z 5935.893) bound to the protein. A mixture of coumarin labeled protein was observed with varying numbers of coumarin bound to

the protein, which was expected. It has been shown that a mixture of different conjugates with a varying number of bound labels for FITC and biotin labeled protein samples are observed by MALDI-MS [27]. It was expected that up to three coumarin molecules could be bound to insulin, and this was observed by UV MALDI-MS. It is important to note that there is no unlabeled protein (MW 5.7 kDa) present in the spectrum, which showed that the labeling procedure was successful and efficient. The low mass range was monitored to ensure that no free fluorophore was present in the sample. Therefore, MALDI-MS demonstrated that the labeling and separation of labeled insulin were efficient.

The fluorescently labeled protein sample was then spotted onto a MALDI plate without any additional matrix, and analyzed by UV FALDI-MS. The covalently bonded coumarin absorbs the UV light from the laser and assists in the soft ionization and desorption of coumarin labeled insulin. UV FALDI-MS spectrum of coumarin labeled insulin (Figure 4b) shows the base peak at m/z 6339.624, which corresponds to three coumarin molecules being bonded to insulin. There are also less intense peaks that correspond to one coumarin (m/z 5935.648) and two coumarin molecules (m/z 6137.631) bound to insulin. Some differences and similarities are observed when comparing UV MALDI (Figure 4a) and UV FALDI-MS (Figure 4b) spectra of fluorescently labeled insulin. The ion intensity is greater in the case of UV MALDI-MS spectra. However, under the same LDI conditions, better mass accuracy and mass resolution for the analysis of fluorescently labeled insulin were achieved in the case of the UV FALDI-MS. Both techniques are able to analyze fluorescently labeled proteins effectively.

Cytochrome C (MW~12.3 kDa) was then labeled using two different labeling protocols: a standard labeling protocol [10] and the new protocol described in the Experimental section. For the standard labeling procedure, 2 μ L of coumarin solution ($c = 10$ mg/mL) in DMSO was added

to 10 μ L of protein solution ($c = 10$ mg/mL) and incubated for 3 hours at room temperature. Figure 5a demonstrates that up to 16 cytochrome C labeling sites (out of 19) were detected using this procedure, with the base peak containing ten coumarin labels. Considering that linear ion mode was used for mass analysis, mass resolution of this coumarin labeled cytochrome C sample was satisfactory.

The MALDI-MS spectrum of cytochrome C, which was labeled using a standard labeling protocol, was then compared to the UV MALDI and FALDI-MS spectra of coumarin labeled cytochrome C which was labeled using the protocol presented in Experimental section. Prior to these experiments, free fluorophore was removed from the protein sample by filtration. Figure 5b shows the UV MALDI-MS spectrum of coumarin labeled cytochrome C which contains a broad, unresolved base peak with the maximum corresponding to 15 coumarin labels. Protein labeling and sample preparation protocols had a large effect on the quality of MALDI-MS spectra shown in Figures 5a and 5b. Higher amounts of coumarin dye and DMSO improved the labeling efficiency of cytochrome C, but the crystallization of the sample with the matrix was diminished. This perhaps led to increased matrix adduct formation during MALDI, which resulted in decreased intensity and unresolved peak in MALDI-MS spectrum of fluorescently labeled cytochrome C (Figure 5b). However, the UV FALDI-MS spectrum of coumarin labeled cytochrome C (Figure 5c) shows peaks that contain between 12 and 18 coumarin labels and mass resolution in this spectrum is better than in the MALDI-MS spectrum of this sample (Figure 5b). There are 19 coumarin labeling sites (i.e., 19 lysine residues) in cytochrome C, so all labeling sites of the protein were not labeled after the two hours of incubation. If the sample was allowed to incubate for longer times (overnight) without the addition of any extra coumarin then all 19 sites of cytochrome C could be observed as labeled (data not shown). Both UV FALDI-MS

(Figure 5c) and UV MALDI-MS of the sample labeled using standard protocol (Figure 5a) can resolve the coumarin labeled cytochrome C ions. However, with the new labeling protocol, almost all of the lysines of cytochrome C were detected with a coumarin bound. The increased labeling efficiency in less time (e.g., 2 hours vs. 3 hours for the two labeling procedures used) may be beneficial for the analysis of fluorescently labeled proteins. This experiment also demonstrated that both coumarin labeling procedures and FALDI-MS can be performed on proteins containing multiple bonding sites that are larger than insulin.

In addition to peptides and proteins, other types of fluorescently labeled biomolecules can be analyzed using UV FALDI-MS. The oligosaccharide, maltoheptaose (MW 1153.00), was labeled with the fluorophore 2-AB, which has an absorption maximum at 330 nm (Table S1), using a labeling kit (Prozyme). The sample was then spotted and analyzed by FALDI-MS in the absence of a matrix since no free 2-AB fluorophore was detected. The FALDI-MS spectrum of 2-AB labeled maltoheptaose is shown in Figure S4 and the base peak in this spectrum is the sodiated ion of maltoheptaose (m/z 1295.571). Considering that 2-AB has been used for fluorescent labeling of O- and N-linked glycans [36], FALDI-MS may be useful for the analysis of more complex carbohydrates. As shown previously [31], glycosphingolipids labeled with NBD fluorophore can be analyzed using UV LDI-MS. Figure S5 shows the FALDI-MS spectrum of the fluorescently labeled lipid, NBD-cholesterol (LOD ~ 250 pmol). NBD is a fluorophore that has an absorption maximum at 465 nm and a less intense absorption peak at 333 nm so it is able to absorb UV light. The base peak in the spectrum is the sodiated ion (m/z 769.534). Since some fragments were observed in the FALDI-MS spectra of NBD-cholesterol and maltoheptaose, finding more suitable fluorophores for labeling and for LDI-MS of these samples would be beneficial.

Fluorescence labeling requires careful optimization of the sample preparation conditions and such derivatization is not necessary for mass spectrometric analyses of most biomolecules. However, fluorescent labeling can improve ionization efficiency of biomolecules, and enable their sequencing and selective ionization in MALDI and FALDI-MS experiments [22-26,31,32]. It appears that widely used laser dyes (e.g., coumarin and rhodamine derivatives), are the best suited for the soft ionization and consequent analyses of biomolecules, such as peptides and proteins, using FALDI-MS [32]. This can be explained by high absorption coefficients and high stability of these fluorescent dyes, which provide efficient light absorption and the stability of derivatized biomolecules during UV and visible-wavelength LDI. High fluorescence quantum yields of laser dyes in the solution and likely solid states may also play a role in efficient energy transfer and soft ionization of derivatized biomolecules since thermal relaxation and decomposition of fluorescent molecules are minimized. Nevertheless, although it likely shares many similarities with the mechanisms of LDI and MALDI [2,19,20,37-40], detailed mechanism of FALDI remains to be determined by analyzing structurally-diverse fluorescently labeled biomolecules using LDI-MS and fluorescence techniques.

Another challenge will be to apply FALDI-MS for specific analyses of biomolecules in complex samples. Present work demonstrates that purified biomolecules can be fluorescently derivatized and analyzed by FALDI-MS and FALDI-MS/MS. For unknown proteins containing single or multiple binding sites, fluorescence labeling could be followed by affinity purification and enzymatic digestion of the protein for further FALDI-MS characterization of its molecular weight, sequence, and fluorophore-binding sites. Alternatively, specific labeling of a biomolecule with a fluorophore (e.g., chemically, metabolically, or genetically) in a complex sample could enable direct and selective FALDI-MS analyses of fluorescently labeled

biomolecules of interest. Therefore, future studies should focus on the development of the protocols for more efficient and selective labeling of biomolecules in complex biological samples (e.g., cells and tissues) for consequent FALDI-MS analyses.

Conclusions

FALDI-MS is a technique that uses covalently-bonded fluorescent dyes for direct LDI-MS analyses of fluorescently labeled biomolecules. FALDI-MS and MS/MS analyses of fluorescently labeled biomolecules can be performed using both visible-wavelength and UV lasers enabling soft ionization of the samples, even with moderate light absorption by a fluorophore at the wavelength used. UV FALDI-MS/MS spectra of peptides labeled with rhodamine B and FAM show an abundance of *a* and *b* fluorophore-containing fragment ions, which can facilitate easier sequencing of labeled peptides than unlabeled peptides. Importantly, relatively big fluorescently labeled proteins (e.g., cytochrome C) can be analyzed using FALDI-MS. The results shown in this study demonstrate that UV FALDI-MS can be used for soft LDI-MS analyses of several types of fluorescently labeled biomolecules such as peptide, protein, lipid, and oligosaccharide samples.

Acknowledgements

This work was supported by the research funds from the University of Toledo (DI). The funding for the acquisition of the UV MALDI-MS instrument was obtained from the National Science Foundation (award # 0923184). The authors thank Yang Xu for the initial work on the MALDI-MS of coumarin labeled cytochrome C.

References

- [1] M. Karas, D. Bachmann, F. Hillenkamp, Influence of the wavelength in high irradiance ultraviolet laser desorption mass spectrometry of organic molecules, *Analytical Chemistry* 57 (1985) 2935–2939.
- [2] R.J. Cotter, J.-C. Tabet, Laser desorption mass spectrometry: mechanisms and applications, *International Journal of Mass Spectrometry and Ion Physics* 53 (1983) 151–165.
- [3] D.J. Rousell, S.M. Dutta, M.W. Little, K.K. Murray, Matrix-free infrared soft laser desorption/ionization, *Journal of Mass Spectrometry* 39 (2004) 1182–1189.
- [4] S. Weidner, G. Kühn, J. Friedrich, Infrared-matrix-assisted laser desorption/ionization and infrared-laser desorption/ionization investigations of synthetic polymers, *Rapid Communications in Mass Spectrometry* 12 (1998) 1373–1381.
- [5] J.J. Boon, T. Learner, Analytical mass spectrometry of artists' acrylic emulsion paints by direct temperature resolved mass spectrometry and laser desorption ionization mass spectrometry, *Journal of Analytical and Applied Pyrolysis* 64 (2002) 327–344.
- [6] M. Karas, F. Hillenkamp, Laser desorption ionization of proteins with molecular masses exceeding 10,000 Da, *Analytical Chemistry* 60 (1988) 2299–2301.
- [7] K. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry, *Rapid Communications in Mass Spectrometry* 2 (1988) 151–153.
- [8] R.C. Beavis, T. Chaudhary, B.T. Chait, Alpha-cyano-4-hydroxycinnamic acid as a matrix for matrix-assisted laser desorption mass-spectrometry, *Organic Mass Spectrometry* 27 (1992) 156–158.
- [9] K. Strupat, M. Karas, F. Hillenkamp, 2,5-Dihydroxybenzoic acid—a new matrix for laser desorption ionization mass-spectrometry, *International Journal of Mass Spectrometry and Ion Processes* 111 (1991) 89–102.
- [10] Z. Sun, E.W. Findsen, D. Isailovic, Atmospheric pressure visible-wavelength MALDI-MS, *International Journal of Mass Spectrometry* 315 (2012) 66–73.
- [11] X.K. Hu, D. Lacey, J. Li, C. Yang, A.V. Loboda, R.H. Lipson, Visible wavelength MALDI using coumarin laser dyes, *International Journal of Mass Spectrometry* 278 (2008) 69–74.
- [12] L.C. Chen, D. Asakawa, H. Hori, K. Hiraoka, Matrix-assisted laser desorption/ionization mass spectrometry using a visible laser, *Rapid Communications in Mass Spectrometry* 21 (2007) 4129–4134.

- [13] K. Tang, S.L. Allman, R.B. Jones, C.H. Chen, Comparison of rhodamine dyes as matrices for matrix-assisted laser desorption/ionization mass spectroscopy, *Organic Mass Spectrometry* 27 (1992) 1389–1392.
- [14] C.Y. Yang, X.K. Hu, A.V. Loboda, R.H. Lipson, A Useful binary matrix for visible-MALDI of low molecular weight analytes, *Journal of the American Society for Mass Spectrometry* 21 (2010) 294–299.
- [15] L.J. Romano, R.J. Levis, Nondestructive laser vaporization of high molecular weight single-stranded DNA, *Journal of the American Chemical Society* 113(1991) 9665–9667.
- [16] D.S. Cornett, M.A. Duncan, I.J. Amster, Liquid-mixtures for matrix-assisted laser-desorption, *Analytical Chemistry* 65 (1993) 2608–2613.
- [17] C.J. Smith, S.Y. Chang, E.S. Yeung, Characterization of neutral red as a visible-wavelength MALDI matrix, *Journal of Mass Spectrometry* 30 (1995) 1765–1767.
- [18] D.S. Cornett, M.A. Duncan, I.J. Amster, Matrix-assisted laser desorption at visible wavelengths using a two-component matrix, *Organic Mass Spectrometry* 27 (1992) 831-832.
- [19] P. D. Shetz, R. Knochenmus, Exciton mobility and trapping in a MALDI matrix, *Journal of Physical Chemistry A* 109 (2005) 4030-4037 .
- [20] T.W. Jaskolla, M. Karas, Using fluorescence dyes as a tool for analyzing the MALDI process, *Journal of the American Society for Mass Spectrometry* 19 (2008) 1054–1061.
- [21] H. Khalfan, R. Abuknesha, M. Rand-Weaver, R.G. Price, D. Robinson. Amino methyl coumarin acetic acid: a new fluorescent labeling agent for proteins, *Histochemical Journal* 18 (1986) 497-499.
- [22] A. Pashkova, E. Moskovets, B.L. Karger, Coumarin tags for improved analysis of peptides by MALDI-TOF MS and MS/MS. 1. Enhancement in MALDI MS signal intensities, *Analytical Chemistry* 76 (2004) 4550–4557.
- [23] A. Pashkova, H-S. Chen, T. Rejtar, X. Zang, R. Giese, V. Andreev, E. Moskovets, B.L.Karger, Coumarin tags for analysis of peptides by MALDI-TOF MS and MS/MS.2. Alexa Fluor 350 tag for increased peptide and protein identification by LC-MALDI-TOF/TOF MS, *Analytical Chemistry* 77 (2005) 2085–2096.
- [24] M. Nakagawa, T. Yamagaki, H. Nakanishi, Fluorescent modification for peptide sequencing by postsource decay-matrix assisted laser desorption/ionization-mass spectrometry, *Electrophoresis* 21 (2000) 1651-1652.
- [25] G. Chiappetta, S. NDiaye, E. Demey, I. Haddad, G. Marino, A. Amoresano, J. Vinh, Dansyl-peptides matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) and tandem mass spectrometric (MS/MS) features improve the liquid chromatography/MALDI-

MS/MS analysis of the proteome, *Rapid Communications in Mass Spectrometry* 24 (2010) 3021-3032.

[26] R. Ullmer, A. Ptematl, A. Rizzi, Derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate for enhancing the ionization yield of small peptides and glycopeptides in matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry, *Rapid Communications in Mass Spectrometry* 20 (2006) 1469-1479.

[27] J. Lu, R. Zenobi, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for identifying the composition of proteins, *Analytical Biochemistry* 269 (1999) 312-316.

[28] B. Salih, R. Zenobi, MALDI mass spectrometry of dye-peptide and dye-protein complexes, *Analytical Chemistry* 70 (1998) 1536-1543.

[29] M. Zehl, G. Allmaier, Investigation of sample preparation and instrumental parameters in the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of noncovalent peptide/peptide complexes, *Rapid Communications in Mass Spectrometry* 17 (2003) 1931-1940.

[30] S.D. Friess, J.M. Daniel, R. Hartmann, R. Zenobi, Mass spectrometric noncovalent probing of amino acids in peptides and proteins, *International Journal of Mass Spectrometry* 219 (2002) 269-281.

[31] M. Landoni, V.G. Duschak, R. Erra-Balsells, A.S. Couto, UV-MALDI mass spectrometry analysis of NBD-glycosphingolipids without an external matrix, *Journal of the American Society for Mass Spectrometry* 19 (2008) 923-926.

[32] R.E. West III, E.W. Findsen, D. Isailovic, Fluorophore-assisted laser desorption/ionization-mass spectrometry (FALDI-MS), *International Journal of Mass Spectrometry* 353 (2013) 54-59.

[33] D. Suckau, A. Resemann, M. Schuerenberg, P. Hufnagel, J. Franzen, A. Holle, A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics, *Analytical and Bioanalytical Chemistry* 376 (2003) 952-965.

[34] J.D. Dunn, J.A. Siegel, J. Allison, Photodegradation and laser desorption mass spectrometry for the characterization of dyes used in red inks, *Journal of Forensic Sciences* 48 (2003) 1-6.

[35] M. Brinkley, A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents, *Bioconjugate Chemistry* 3 (1992) 2-13.

[36] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid, *Analytical Biochemistry* 230 (1995) 229-238.

[37] F. Hillenkamp, T. W. Jaskolla, M. Karas: The MALDI process and method, in “MALDI-MS: A Practical Guide to Instrumentation, Methods and Applications”, second edition, F. Hillenkamp and J. Katalinic (eds.), Wiley-VCH, Weinheim, Germany, (2013) 1-40.

[38] R. Cramer, K. Dreisewerd, UV matrix-assisted laser desorption ionization: principles, instrumentation, and applications, in The Encyclopedia of Mass Spectrometry, Vol. 6: ionization methods, M. L. Gross, R. M. Caprioli (eds.), Elsevier Science, Amsterdam, NL. (2007) 646-661.

[39] K. Dreisewerd, The desorption process in MALDI, Chemical Reviews 103 (2003) 395-426.

[40] “Laser Ionization Mass Analysis” A. Vertes, R. Gijbels, F. Adams (eds.) John Wiley & Sons, Inc., New York, NY (1993)

Figure Captions

Figure 1. UV FALDI-MS spectrum of rhodamine B-GILGFVFTL.

Figure 2. (a) UV FALDI-MS spectrum of FAM-angiotensin II. (b) UV FALDI-MS spectrum of FAM-bombesin.

Figure 3. UV MALDI-MS spectrum of coumarin labeled bradykinin with the matrix CHCA.

Figure 4. (a) UV MALDI-MS spectrum of coumarin labeled insulin with sinapinic acid as the matrix. (b) UV FALDI-MS spectrum of coumarin labeled insulin. The $\Delta m/z$ 202 corresponds to the mass of a protein-bonded coumarin molecule.

Figure 5. (a) UV MALDI-MS spectrum of cytochrome C labeled with coumarin using a standard labeling protocol (b) UV MALDI-MS spectrum of cytochrome C labeled with coumarin using new labeling protocol (c) UV FALDI-MS spectrum of cytochrome C labeled with coumarin using new labeling protocol. The $\Delta m/z$ 202 corresponds to the mass of a protein-bonded coumarin molecule.

Figures

Figure 1. UV FALDI-MS spectrum of rhodamine B-GILGFVFTL.

Figure 2. (a) UV FALDI-MS spectrum of FAM-angiotensin II. (b) UV FALDI-MS spectrum of FAM-bombesin.

Figure 3. UV MALDI-MS spectrum of coumarin labeled bradykinin with the matrix CHCA.

Figure 4. (a) UV MALDI-MS spectrum of coumarin labeled insulin with sinapinic acid as the matrix. (b) UV FALDI-MS spectrum of coumarin labeled insulin. The $\Delta m/z$ 202 corresponds to the mass of a protein-bonded coumarin molecule.

Figure 5. (a) UV MALDI-MS spectrum of cytochrome C labeled with coumarin using a standard labeling protocol (b) UV MALDI-MS spectrum of cytochrome C labeled with coumarin using new labeling protocol (c) UV FALDI-MS spectrum of cytochrome C labeled with coumarin

using new labeling protocol. The $\Delta m/z$ 202 corresponds to the mass of a protein-bonded coumarin molecule.

Graphical Abstract