On-Tissue N-Terminal Peptide Derivatizations for Enhancing Protein Identification in MALDI Mass Spectrometric Imaging Strategies

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Matrix-assisted laser desorption/ionization (MALDI) is a new tool that can acquire the localization of various compounds, including peptides and proteins, directly from tissue sections. Despite the important developments recently performed in the field of MALDI imaging in tissue, the precise identification of compounds still needs improvement. We have developed N-terminal chemical derivatization strategies to improve tissue identification of proteins, including de novo sequencing performance. We have first focused on sulfonation agents, such as 4-SPITC and 3-SBASE. These two derivatizations were optimized to be performed directly on tissue sections. By adding a negative charge at the N-terminus of a tryptic digest peptide, we were able to generate a complete y fragment series directly from the tissue. Of these derivatizations, 3-SBASE has shown to be more efficient, as loss of the derivative group is one of the major fragmentation pathways for 4-SPITC. 3-SBASE was optimized so that the derivatization reaction could be automatically performed using an automatic microspotting device. It was then included in an automatic process that included automated trypsin digestion and matrix deposition. Derivatizations allowed the acquisition to be easily interpretable by MS^2 spectra, leading to very precise identification as well as easy manual reading of sequences for de novo sequencing. It was observed that only arginine-terminated peptides were observed after derivatization, likely due to the high gas-phase basicity of such peptides compared to those that are lysine-terminated. We also observed a stop in the y fragmentation series for peptides presenting a miscleavage. We have now begun to study a different derivatization using *N*-succinimidyloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP). This derivatization allows the orientating of a fragmentation toward a series of fragment ions, and thus it is independent of the presence of basic residues in the sequence. This derivatization can be performed at room temperature, which greatly facilitates the automation of the process. The TMPP derivatization therefore yields an advantageous new generation of derivatives suited for use in tissue.

Since its introduction in 1997 by the group of Caprioli,[1] direct tissue analysis and imaging by mass spectrometry (MS) has become a favorable alternative to classical techniques. Directly analyzing tissues gives access to the detection of hundreds of biomolecules while maintaining tissue integrity and molecular localization.[2−5] By automating this approach, molecular images of the distribution of biomolecules can be obtained in a single-step analysis.[6−12] Recent applications demonstrate the potential of such technology, in particular for pathology studies.[13−16] This tool is well-suited to facilitate biomarkers research, to better understand molecular mechanisms implicated in diseases, and to track drugs within tissues.[17−23]

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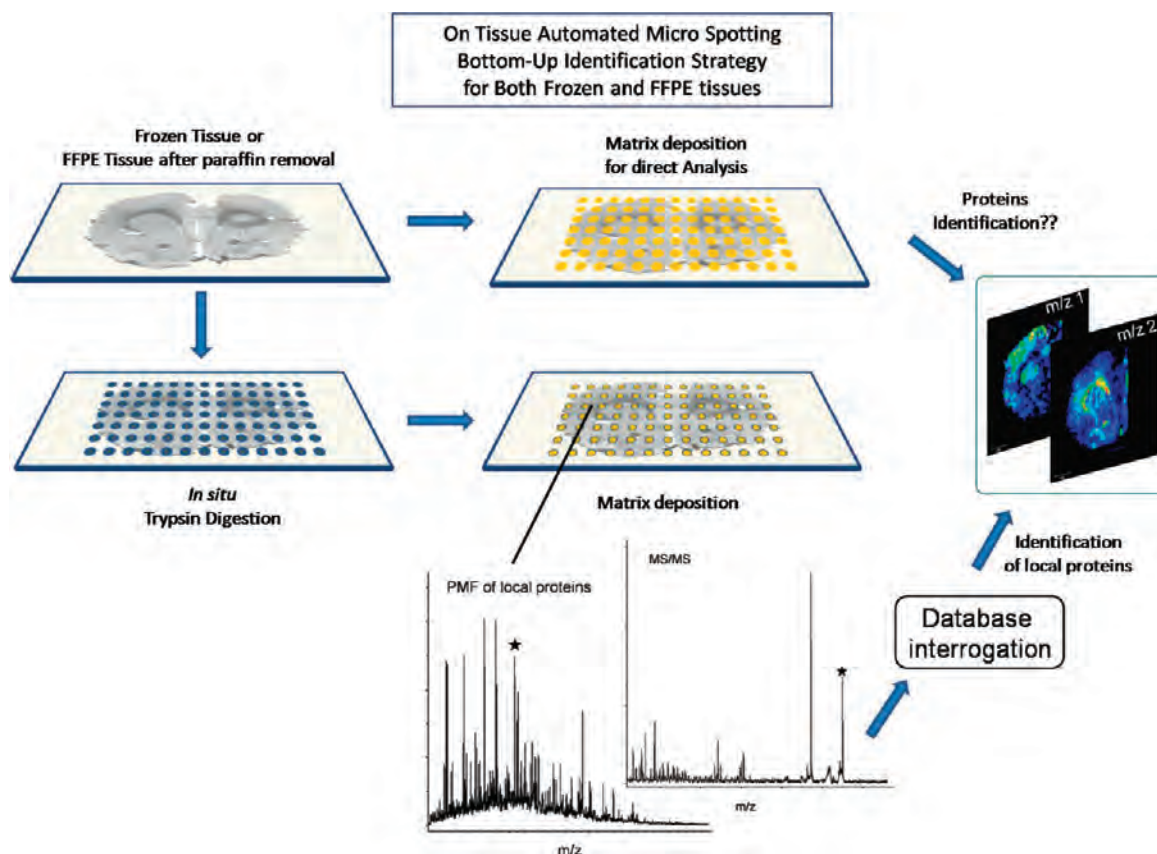
Figure 1. Schematic workflow of MALDI-MSI "bottom-up" strategy used to perform protein identification.

With the use of matrix-assisted laser desorption/ionization (MALDI), molecular data on a wide range of compounds (peptides, proteins, lipids, sugars, etc.) can be retrieved with localization using limited cellular levels. The correlation of molecular images to histological features highlights markers of interest. Conversely, MALDI imaging of known compounds adds another dimension to our knowledge of biological processes by accessing the spatial distribution of peptides and proteins. Thus, the localization of molecules of interest can be obtained; however, identification of these specific biomolecules remains to be performed. The most straightforward method of identification is to return to classical proteomic strategies using extraction, prepurification, and separation of peptides/proteins prior to their identification. Such strategies, however, are time- and sample-consuming and can lead to peptide/protein modifications if reagents degrade and/or chemically modify the compounds. Moreover, to access high-throughput identification of markers and to correlate them with a histological feature, it is necessary to develop tissue identification strategies. Such strategies are also of significant interest for peptides/proteins that are extremely localized in a tissue section, making them difficult to retrieve using a global extraction approach because of their dilution over the whole sample. Because MALDI does not generate multiply charged ions, currently, tissue identification of proteins is not directly accessible by "top-down" approaches. It is therefore necessary to dispose of peptides by developing "bottom-up" strategies. The principle of "bottom-up" strategies is to generate peptides from proteins by enzymatic digestion directly at the tissue level (Figure 1). "Bottom-up" strategies for MALDI mass spectrometric imaging (MALDI-MSI) were introduced two years ago, demonstrating a good ability to generate digestion fragments up to m/z 3 500. This approach allows both the acquisition of images of proteins based on their digestion peptides (if suited deposition methods are used) as well as the retrieval of structural information of the proteins by performing MS^2 experiments on the peptides. Identifying endogenous peptides or proteins directly from tissue sections has shown to be more difficult compared to on target preparation. In fact, we can assume that the tissue section underneath the matrix layer could decrease the internal energy of the ions formed in the gas phase by allowing part of the energy resulting from photon excitation to be evacuated (i.e., thermal energy). Moreover, the insulating nature of the tissue section and its thickness render MS/MS parameters more difficult to control, especially when the instrument is an axial MALDI time-of-flight (MALDI-TOF) mass spectrometer. Thus, in most cases, MS/MS is used to verify the sequence of an expected endogenous peptide or digestion peptides, and few examples are found where in-tissue MS/MS was used to identify unidentified markers. In addition, MS/MS spectra generated from instruments equipped with a MALDI ion source are often more complex to analyze because of the different types of fragment series generated during the fragmentation process. This is especially true on MALDI-TOF systems that frequently have weak fragmentations (especially in the higher

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m/z range of the MS/MS spectrum) and a significantly different series of fragment ions. These features can consequently create a largely incomplete set and only allow the observation of small sequence tags. The sequencing of peptides can be very difficult to establish due to the different fragmentation ions observed during MS/MS experiments, such as immonium ions,[24] internal fragments, N-terminal ions,[25,26] or C-terminal ions.[27] A nomenclature of these ions has been proposed by Roepstorff and Fohlman.[28]

Even using classical identification strategies, many efforts have been made to simplify data interpretation (especially for MALDI systems) by increasing fragmentation ion yield and orienting fragmentations toward a specific series of fragment ions. In this respect, derivatization at the C- or N-terminal part of peptides by adding positive or negative charges has shown to be an efficient strategy,[29] especially for post source decay (PSD) spectra. In general, N-terminal modifications are easier because of amine reactivity, and they have been shown to be more efficient for fragmentations. Sulfonation at the N-terminal part of peptides appears to be especially advantageous when working on tryptic digestion peptides that are naturally positive at their C-terminal part. Keough and co-workers have shown that if a negatively charged group is added at the N-terminus of tryptic peptides using chlorosulfonylactetyl chloride[30,31] or 2-sulfobenzoic acid cyclic anhydride,[32] it is possible to orientate fragment ions toward y_i^+ ions, thereby allowing de novo sequencing to be performed.[33] This method was later improved by using 3-sulfopropionic acid *N*-hydroxysuccinimide (NHS) ester as the derivatization agent,[34] allowing the reaction to be performed in the aqueous phase. This reagent is fast and water compatible, and it has now been marketed[35] and is quite expensive. Alternatively, 4-sulfophenyl isothyocyanate[36−38] was proposed for derivatization, allowing liquid chromatography[39] and quantification with isotope coding[40] to be performed. These derivatizations are simple and can be performed quickly, showing good reaction yields, and

they often lead (depending on the peptide sequence) to the loss of the derivative group as a major fragmentation pathway. In this context, the benefits of derivatization are lowered by the recovery of the native peptide ion. More recently, another water compatible reagent, 3-sulfobenzoic acid NHS ester, was proposed.[41] This sulfonation agent presents more efficiency, since no loss of the derivative group is observed. This also results in the observation of a complete y ion series. Derivatization by addition of a positive charged at the N-termini part of the peptides was also studied. In particular, derivatization with *N*-succinimidyloxycarbonylmethyl)-tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP), introduced by Huang et al.,[42] was successfully used for peptide sequence identification. Derivatization using TMPP orients fragmentation toward N-terminus fragment ions, with a strong a_i^+ series of fragment ions and only a few b_i^+, c_i^+, and d_i^+ fragment ions. For this derivatization, fragmentation orientation is independent of the presence and/or position of basic amino acid residues in the peptide sequence, which makes it of wider use.

In this study, we have investigated N-terminal chemical derivatization of peptides in tissue for protein identification using bottom-up imaging strategies. Derivatization of digestion peptides at the tissue level allows for the orientation of fragmentation patterns toward a specific series of fragment ions and eases peptide identification. We have focused our study on two types of derivatizations. First, we have studied sulfonation agents such as 4-sulfophenyl isothiocyanate and sulfobenzoic acid succinimidyl ester, giving orientation toward y_i^+ fragments for peptides presenting a basic amino acid residue at their C-termini (i.e., tryptic digestion peptides). We have also investigated derivatization independent of the presence of a basic residue at the N-termini part of peptides, such as TMPP derivatization. Studies and optimizations of reactions were first performed at the whole tissue section scale. Protocols were then modified and adapted to allow derivatization to be performed at the microscale level using MALDI-MSI, with automation, if required. This was achieved using a microspotting piezoelectric automatic deposition device. Here, we report that in-tissue derivatization is a highly efficient strategy for protein identification in MALDI-MSI and can be performed by automatic derivatization at the microscale level. This is a key point for direct and confident in-tissue identification of markers using MALDI-MSI.

EXPERIMENTAL PROCEDURES

Materials. 4-Sulphophenyl isothiocyanate (4-SPITC), 3-sulfobenzoic acid (3-SBA), dicyclohexylcarbodiimide (DCC), NHS, α-cyano-4-hydroxycinnamic acid (HCCA), trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), acetone, acetonitrile (ACN), aniline (ANI), triethylamine (TEA), and TMPP were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and used as received. The trypsin enzyme was from Promega France.

Chemical Synthesis of 3-Sulfobenzoic Acid Succinimidyl Ester (3-SBASE). The reagent was synthesized according to a previously established protocol.[41] Briefly, 1.0 g of 3-SBA was

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completely dissolved in 10 mL of DMSO. A 1.5 M excess of DCC and equimolar amount of NHS were then added to the solution, and the mixture was stirred overnight at room temperature. The precipitated DCU, a side product of the reaction, was then filtered and discarded. The remaining solution was filtered to remove side products. After filtration, 60 mL of cold acetone (4 °C) was slowly added to precipitate 3-sulfobenzoic acid succinimidyl ester (3-SBASE), which was then filtered and washed four times with cold acetone and then dried.

Tissue Preparation. Thin 10 μm tissue sections were obtained from frozen rat brains using a cryostat (Leica, Germany) and applied onto indium−tin oxide (ITO)-coated conductive glass slides (Bruker Daltonics, Bremen, Germany). Tissue sections were then submitted to a washing step using 70% ethanol and 95% ethanol for 30 s each, followed by a washing step using chloroform for 30 s to partially remove abundant phospholipids, as described by Lemaire et al.[43]

On-Tissue Digestion.

Whole tissue trypsin digestions were performed by applying a solution of trypsin (20 μg/mL) in pure H_2O using a micropipet. Tissue sections were then incubated for 1 h at 37 °C in an atmosphere saturated with $MeOH/H_2O$ (1:1, v/v).

Automatic trypsin digestions were performed using a high accurate position chemical inkjet printer (CHIP-1000, Shimadzu Biotech, Kyoto, Japan). The printed array of the CHIP-1000 on the tissue section was composed of spots spaced 250 μm center-to-center. A total of 20 nL of solution containing 25 μg/mL of trypsin in pure H_2O was applied to each spot. Five droplets of approximately 100 pL were deposited on each spot per cycle. A total of 40 iterations were thus necessary to obtain the final volume. Tissue sections were then incubated at 37 °C for 2 h in a box saturated in $MeOH/H_2O$ (1:1, v/v).

On-Tissue Derivatization. *4-SPITC Derivatization.* 4-SPITC derivatization was performed manually on the whole tissue section. Manual derivatization was performed by depositing a solution of 4-SPITC (10 mg/mL) in 25 mM NH_4HCO_3 buffer on the tissue section. The tissue was then incubated in a box saturated in $MeOH/H_2O$ (1:1, v/v) at 50 °C for 1 h. The tissue was then allowed to dry at room temperature, and a solution of 1% TFA was deposited to improve ionization of derivate peptides prior to matrix deposition.

3-SBASE Derivatization. 3-SBASE derivatization was either performed manually on the whole tissue section or automatically using the microspotting device. Manual derivatization was performed by depositing a solution of 3-SBASE (20 mg/mL) in 25 mM NH_4HCO_3 buffer on the tissue section. The tissue was then incubated in a box saturated in $MeOH/H_2O$ (1:1, v/v) at 50 °C for 1 h. The tissue was then allowed to dry at room temperature, and a solution of 1% TFA was deposited to improve ionization of derivate peptides prior to matrix deposition.

TMPP Derivatization. For TMPP derivatization, a solution of 1 mg/mL in ACN/H_2O (3:7, v/v) was deposited using a micropipet on the whole tissue section. The section was then left until the complete solution was dried. A solution of ACN/H_2O containing 1% of TEA was then deposited using a micropipet and incubated for 1 h at room temperature.

Automatic derivatization was performed using the automatic microspotting device. The CHIP-1000 was used to deposit TMPP on the tryptic spots according to the same procedure previously described for trypsin digestion. A total of 10 nL of solution containing 1 mg/mL of TMPP in ACN/H_2O (3:7, v/v) was deposited at each spot. Five droplets of approximately 100 pL were deposited at each spot per cycle. Twenty iterations were thus necessary to obtain the final volume on each spot. A solution of ACN/H_2O containing 1% of TEA was then spotted, initiating the derivatization reaction. The automated microspotting was performed either according to a global square area of points or by dividing this area into smaller squares, each including 10-by-10 spots to increase the reaction yield.

Imaging Mass Spectrometry of Proteins. A solid ionic SA/aniline matrix was used to image the proteins. The matrix solution was deposited using a chemical inkjet printer CHIP-1000. A total of 20 nL of ionic matrix solution, containing 10 mg/mL of SA and 8.1 μL of aniline in 0.1% ACN/TFA (6.4, v/v), was deposited at each spot. The printed array on the tissue section was composed of spots spaced by 250 μm center-to-center. The images were acquired using an UltraFlex II MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a Smart-beam laser, with a repetition rate up to 200 Hz[44] controlled by FlexControl 3.0 software (Bruker Daltonics, Bremen, Germany). Images were performed in the positive linear mode, and MALDI-MS spectra were acquired in the m/z range from 2000 to 35 000. A total of 300 spectra were acquired at each spot at a laser frequency of 100 Hz. The images were recorded and reconstructed using Fleximaging 2.1 (Bruker Daltonics, Bremen, Germany) software.

Imaging Mass Spectrometry of Peptides. Solid ionic matrix HCCA/aniline was used as the matrix for imaging experiments of peptides and was prepared following a previously established procedure.[45] Matrix solution was deposited using the CHIP-1000 printer. A total of 40 nL of ionic matrix solution containing 10 mg/mL of HCCA and 7.2 μL of aniline in 0.1% ACN/TFA (6:4, v/v) was spotted at each spot. Five droplets of approximately 100 pL were deposited at each spot per cycle. A total of 20 iterations were thus necessary to reach the total final volume. The images were acquired using the UltraFlex II MALDI-TOF/TOF instrument. Images were performed in the positive reflectron mode, and MALDI-MS spectra were acquired in the m/z range from 550 to 5000. A total of 300 spectra were acquired at each spot at a laser frequency of 100 Hz. The images were recorded and reconstructed using the Fleximaging 2.1 software.

MS/MS of Digested and Derivatized Tissues. MALDI-TOF MS/MS experiments on digested and derivatized tissue sections were performed using the Ultraflex II TOF−TOF instrument equipped with LIFT III cells. For MS/MS experiments, parameters were set as follows: the laser repetition rate was 100 Hz with 33% attenuation; ion source voltages were 8 and 7.3 kV on the MALDI sample plate and first electrode, respectively; the LIFT cell was pulsed from ground to 19 kV for electrodes 1 and 2, and in the last step, electrode 3 was decrease to 3.2 kV; the reflector end voltage was set to 29.5 kV; the midgrid was set to 13.85 kV. For

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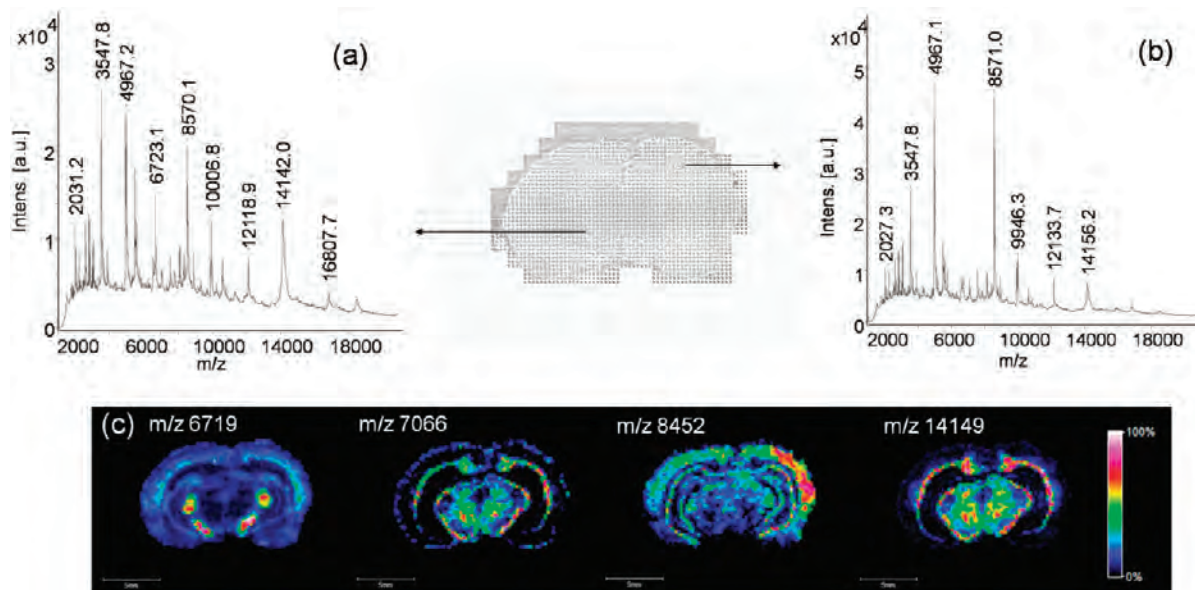
Figure 2. (a and b) MALDI MS spectra recorded in two different locations of a rat brain tissue section after automated microspotting of matrix solution (SA/ANI) and (c) reconstructed molecular images for *m/z* 6719, 7066, 8452, and 14149.

each MS/MS spectrum, 5000 total shots were averaged, including 1000 for parent ions and 4000 for fragments.

Data Analysis. Protein identification in databanks was performed using the Biotools 3.0 software (Bruker Daltonics, Bremmen, Germany) connected to the Mascot search engine. The NCBI, Swissprot, or ESTs databanks with oxidation as variable modification and no fixed modification were also used. Taxonomy was specified to be *Rattus norvegicus*. Trypsin was selected as an enzyme, and two missed cleavages were used. The mass tolerance was set at 1 and 0.5 Da for the MS and MS/MS, respectively. For derivatized peptides, the mass of the parent ion used was the mass without the N-terminal derivatization, taking into account that C-terminal fragment ions do not present mass variations after derivatization.

RESULTS

MALDI-MSI of Proteins. Using a strategy aiming to localize and identify proteins, MALDI images of intact proteins were first recorded. Automatic microspotting of the matrix was used in combination with solid ionic matrix SA/ANI, since these matrixes have been shown to be better suited for microspotting compared to classical matrixes. Under these experimental conditions, hundreds of protein signals were observed from the different regions of the tissue section (Figure 2, parts a and b). Sensitivity can be increased by moving within the matrix spot and averaging spectra from different locations. Even though microspotting does not offer the highest spatial resolution (100−150 μm spot size and ~250 μm spot-to-spot center in along *x* and *y*), the observed proteins do present a highly specific distribution in the tissue section, as illustrated in Figure 2c. If protein distribution across the tissue section is obtained, however, it is not possible to obtain information concerning the primary structure of these proteins using direct analysis, and mass measurement is not sufficient to yield identification beyond a doubt. Since top-down strategies are not currently accessible for use with MALDI from mixtures, bottom-up strategies, involving sample digestion, are therefore required. It was previously shown that direct in-tissue enzymatic

digestion is feasible both from frozen[46] and FFPE[16,47−49] tissue sections. Such "MALDI-MSI" strategies allow digestion while keeping the generated peptide localization, when using appropriate systems for the enzyme deposition. It is therefore possible to image proteins based on their digestion fragments and to reach identification of the proteins by performing MS² experiments. Moreover, by combining the spatial distribution of peptides with their identification by MS/MS experiments, the proteins can be more accurately identified, thereby avoiding their extraction and separation.

MALDI-MSI of Digested Proteins and Identification. A MALDI-MSI strategy involving in situ enzymatic digestion and using trypsin was performed to identify proteins contained in the rat brain tissue section. To prevent delocalization of the digested peptides, the enzyme was deposited using the automatic microspotting system. Conditions were optimized to reach the best digestion conditions. The concentration of trypsin was set to 20 μg/mL, and 40 iterations were performed to obtain a total volume of 20 nL per spot, followed by an incubation step for 2 h at 37 °C. This procedure was found to be the more effective, and images of peptides were then obtained by rastering the whole tissue section after deposition of HCCA/ANI according to the same raster of points used for trypsin digestion. Spectra were also recorded on an adjacent tissue section after digestion, except we used a matrix suited for proteins analysis to check out the effectiveness of the digestion. Almost no signals above *m/z* 5000 were observed from these sections, certifying that a major part of the proteins were digested under these conditions. From the digested tissue sections using HCCA/ANI, hundreds of digested

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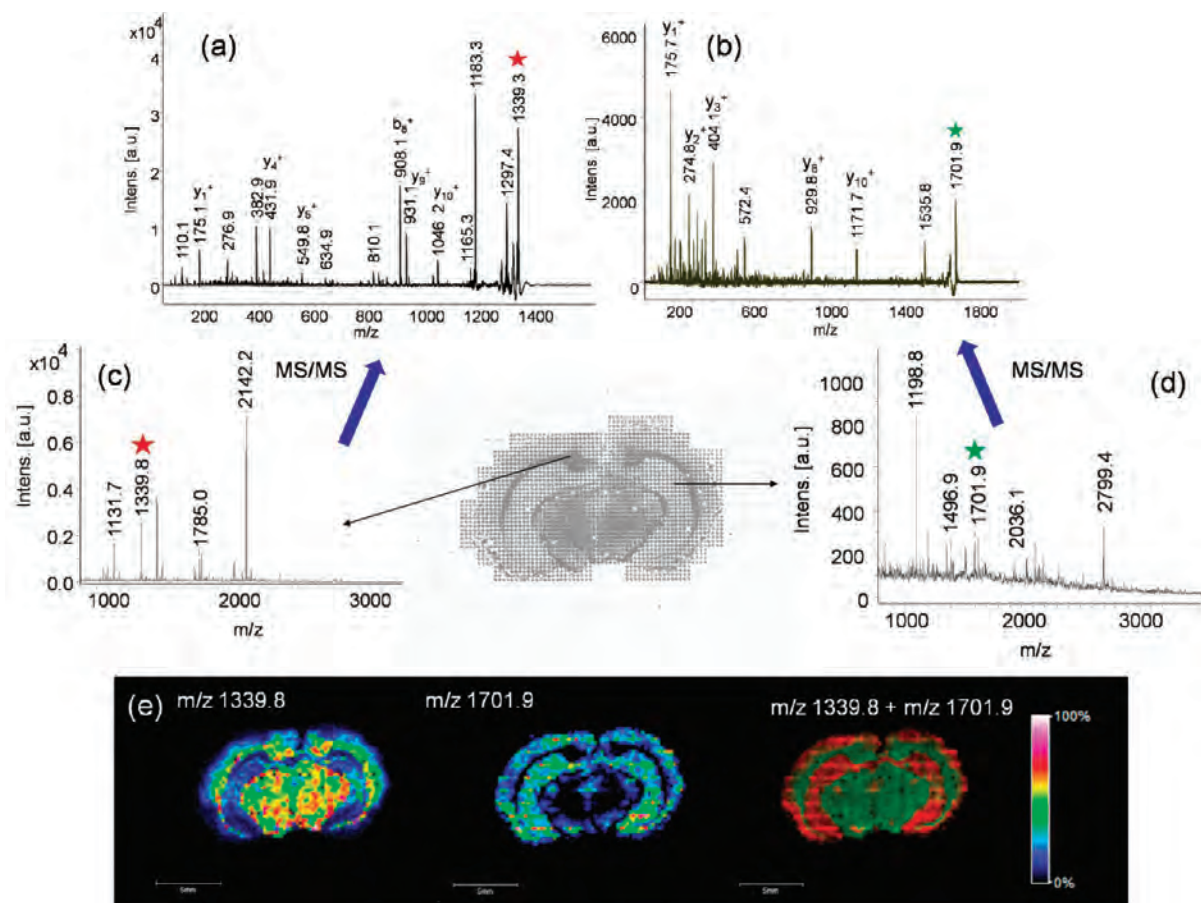
Figure 3. MALDI-MSI of a rat brain tissue section after in-tissue trypsin digestion followed by matrix deposition (HCCA/ANI) using an automated microspotting device. (a and b) MS spectra recorded at two different locations of the tissue section and (c and d) corresponding MS/MS spectra of ions at m/z 1339.8 and m/z 1701.9. (e) Reconstructed molecular images for m/z 1339.8, 1701.9, and colocalized image of both ions.

peptides were detected. Peptide profiles were extremely different according to the position of the spot on the tissue section, reflecting the differences in protein expression according to the cell's phenotype. Figure 3 presents the digestion profiles recorded in the corpus callosum (Figure 3a) and in the thalamic nucleus (Figure 3b). Two ions specific of each region were thus selected from these profiles, m/z 1339.23 for the corpus callosum and m/z 1701.94 for the thalamic nucleus, respectively. For each of these ions, MALDI molecular images were reconstructed (Figure 3c). Molecular images revealed that these ions present an opposing localization in the rat brain. These ions were further studied in MS^2 for identification. The MS^2 spectrum of m/z 1339.23 ion presents a rather poor fragmentation pattern, with only a few intense peaks. This peptide was identified using a databank query, with *R. norvegicus* as the taxonomy, to be a fragment of myelin basic protein (MBP) with a rather poor identification score (score 42, expect 0.0015). On the basis of this identification, postassignment of peaks demonstrated that many different fragment ion types were observed in the spectrum, as expected for MALDI/TOF MS/MS spectra. A few sequence ions were observed, and the y series of ions was clearly incomplete. For such a peptide, de novo sequencing would have been extremely difficult, and still the database identification would not be very reliable. This clearly highlights difficulties of identification using classical strategies. Identical observations can be seen from the MS^2 spectrum recorded for the m/z 1701.94 parent ion. For this ion, more fragments were observed; however,

these fragments were primarily in the low-mass region of the spectrum and had a clear lack of fragment ions in the intermediate m/z range. This again rendered identification difficult. Databank queries allowed for the identification of this peptide as a fragment of the tubulin α protein, but with a relatively poor identification score (score 58, expect 6.2×10^{-5}). For this peptide, the y series of ions was partially observed; however, the fragments were overwhelmed in many other fragments, including internal fragments and immonium ions. Thus, as demonstrated, a strategy simply based on trypsin fingerprint identification is not sufficient, especially when considering that proteins were not separated before digestion. In fact, peptides are generated in tissue from the digestion of hundreds of proteins, requiring high-quality MS^2 spectra to be obtained in order for true identification to be performed. To overcome this drawback, N-terminal chemical derivatizations were employed. N-Terminal derivatizations of peptides are known to orient fragmentation toward a specific series of fragment ions, thus improving the yields of these fragments. 4-SPITC (see the Supporting Information) and 3-SBASE (see the Supporting Information) N-terminal derivatizations were thus evaluated. These derivatizations orient fragmentation toward y_i^+ ion series and should allow for better protein identification. Both of these derivatizations were previously studied by several groups[39,41] on model peptides such as ASHLGLAR, but in our approach, this was performed directly in the tissue section. It was shown that both 4-SPITC and 3-SBASE derivatizations are efficient
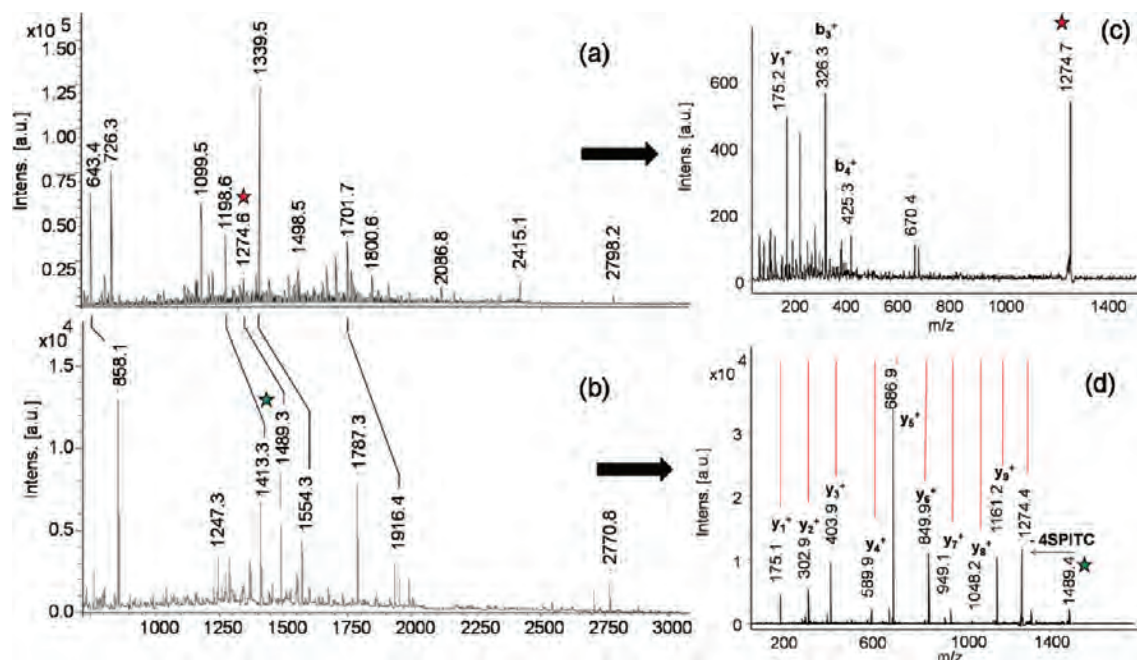
Figure 4. (a and b) MALDI MS spectra recorded on a rat brain tissue section after in-tissue trypsin digestion (a) and in-tissue trypsin digestion followed by derivatization with 4-SPITC (b). (c and d) MALDI MS/MS spectra of one of the peptides (*m/z* 1274.7) from digestion (c) and the same peptide after derivatization with 4-SPITC (*m/z* 1489.9) (d).

and greatly improve MS^2 spectra interpretation by generating a complete and highly predominant y_i^+ ion series. Under such conditions, the peptide's primary structure can even be determined by de novo sequencing with great ease. It should be noted, however, that most of derivatives are lost during the fragmentation, generating an intense fragment and decreasing the global intensity of the y_i^+ ion series. In an ASHLGLAR model peptide, 4-SPITC derivatization leads to the observation of an intense peak, corresponding to the loss of the 4-SPITC group and hindering the observation of the y_i^+ series. For 3-SBASE derivatization, such a loss of the derivative group is not observed, and consequently, the y_i^+ series of fragment ions is observed with higher intensity. Taking into account the behavior of both reagents, 3-SBASE derivatization should be preferred to 4-SPTIC, but both were evaluated for performance on tissue derivatization.

On-Tissue N-Terminal Derivatization for Protein Identification by MALDI Profiling. 4-SPITC and 3-SBASE were used for protein identification, and protocols were optimized for on-tissue derivatization. The more sensitive points concerning within tissue derivatization are the incubation of the reagent on the tissue sections (~50 °C for both derivatizations) and the buffer used for the reaction, since it is not possible to avoid altering compound localization when performing washing or purification of compounds on the tissue sections. Full derivatization of the whole tissue section can be obtained by covering the digested section with 4-SPITC solution and placing it under saturated conditions at 50 °C (Figure 4). It is clearly noticeable that fewer signals with slightly lower general intensities occurred after derivatization, evident from a direct comparison of the spectra recorded immediately after trypsin digestion (Figure 4a) to those collected after trypsin digestion followed by 4-SPITC derivatization (Figure 4b) on the scale of the whole tissue section. This phenomenon, observed for all tissue sections and all areas of the tissue sections,

was previously described by different groups for within solution derivatization.[38] The most reasonable hypothesis is that ionization suppression is due to the presence of sodium, thereby hampering matrix crystallization. It was shown that the use of matrixes containing 2,4,6-trihydroxyacetophenone with diammonium citrate (THAP/DAC) reduced ionization suppression and thus improved detection of 4-SPITC-derivatized peptides.[50] For this reason, THAP matrix containing DAC was tested to improve detection of derivatized peptides in the tissue sections. As expected for tissue analysis using THAP as a matrix, highly abundant lipid signals were observed, but peptide signals were not increased (data not shown). Thus, the classical HCCA matrix was preferred for the remaining experiments. Even with slightly lower intensities, however, derivatized peptides were observed at their expected mass from tissue sections. Within tissue MS/MS experiments performed on the [M + H]$^+$ ion of a tryptic peptide after 4-SPITC derivatization (Figure 4d) showed that addition of 4-SPTIC moiety allowed an increased and orientated fragmentation toward the y_i^+ series of ions, as observed from the MS/MS spectrum with a complete and quite intense y series. In addition, it should be noted that an intense peak corresponding to the loss of the 4-SPITC moiety was observed in the spectrum, as expected. This is a drawback of 4-SPITC derivatization that is commonly observed for within solution derivatization. Despite this, the peptide sequence is easily and beyond a doubt obtained by databank query with a high score (score 103, expect 1.8×10^{-9}); it can be attributed to a hemoglobin subunit β 1 fragment (LLVVYPWTQR). The sequence, however, can also be easily obtained de novo automatically or even manually. Concerning 3-SBASE derivatization, identical general observations similar to those for 4-SPITC were made. Mass spectra recordings in tissue after trypsin digestion (Figure 5a) and after digestion

(50) Oehlers, L. P.; Perez, A. N.; Walter, R. B. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 752–758.
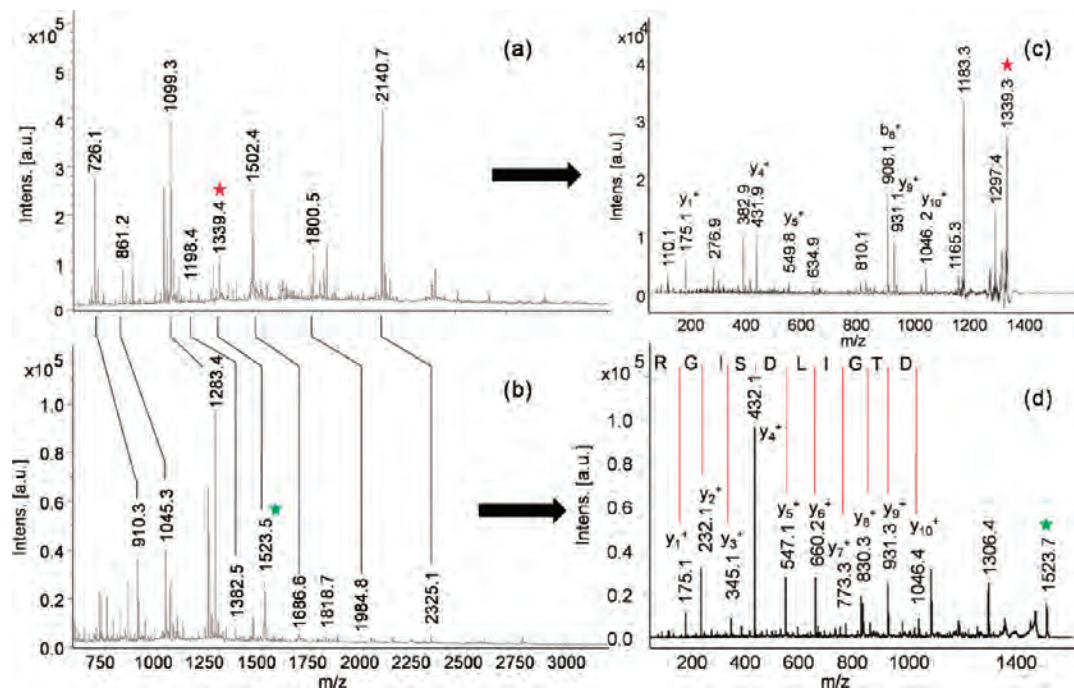
Figure 5. (a and b) MALDI MS spectra recorded on a rat brain tissue section after in-tissue trypsin digestion (a) and in tissue with trypsin digestion followed by derivatization with 3-SBASE (b). (c and d) MALDI MS/MS spectra of one of the peptides generated from digestion (*m/z* 1339.3) (c) and the same peptide after derivatization with 3-SBASE (*m/z* 1523.7) (d).

followed by 3-SBASE derivatization (Figure 5b) showed that a part of the digestion peptides can be directly derivatized from tissue sections. For 3-SBASE, the number of detected peaks and their intensities were higher than for 4-SPITC. This can be attributed to a higher reaction yield, a better compatibility of the buffers used for the reaction, or a formation of derivatized species with higher ionization efficiency. By comparison with the nonderivatized spots, a mass shift of 184 u was observed for many tryptic peptides, proving the efficiency of the reagent to perform N-terminal derivatization directly in tissue. In particular, the peptide found at *m/z* 1339.23, corresponding to a fragment of MBP, and the peptide at *m/z* 1701.94, from tubulin α protein, were both shifted with the expected mass and appear at *m/z* 1523.67 and *m/z* 1902.78, respectively. It should also be noted that derivatized spectra do not contain lysine-terminated peptides, leading to the suppression of signals by the dominant arginine-terminated peptide. For both ions, the MS^2 spectra obtained after derivatization present a very different fragmentation pattern from those without derivatization. As expected, a clear increase of the fragmentation ion yield and orientation of the fragmentation was observed. For both spectra, the most intense and quasi-unique observed fragments were y fragments. For *m/z* 1902.78, the complete y_i^+ series of the fragment ion was observed. This allowed doubtless identification of a fragment of tubulin α (VFVDLEPTVIDEVR) by databank query with a high confidence score (score 142, expect 1.1×10^{-12}). It was also evident from the MS^2 spectrum that this peptide can also be identified using automatic de novo sequencing bioinformatics tools or by manual identification. We must note that, for this particular peptide, the native peptide has an *m/z* of 1701.94 and of 1902.78 after N-terminal derivatization; thus the mass shift is not 184 u., as expected, but 184 + 17 u. The peptide sequence, however, corresponded to the expected sequence for a classically

derivatized peptide. As all observed mass fragments show the expected *m/z*, we must conclude that the modification is a result of the derivative group and does not change the fragmentation properties. For the derivatized peptide at *m/z* 1523.67, a clear y series of fragments was also observed, although in this case the sequence is not fully complete. This peptide, however, can be easily identified as a fragment of MBP (HRDTGILDSIGR) with a high score (score 94, expect 9.6×10^{-9}). Two amino acids were lacking to complete the sequence. The stop in the sequence corresponded to the presence of an arginine residue. This precise peptide contains a miscleavage site, a phenomenon quite often observed for trypsin digestion from tissue sections. This is likely due to a lack of accessibility of the enzyme to some basic residues due to peptide conformation and/or inclusion in the tissue. For all peptides containing a miscleavage, a stop in the y_i^+ series of the ion was observed due to the benefit of N-terminal b_i^+ fragments, such as for the fragment at *m/z* 1092.32 corresponding to the b_8^+ ion. In the present case, the miscleavage is situated at the end of the sequence and does not significantly hamper the identification. If the miscleavage site is in the middle of the rather long peptide sequence, however, identification can be rendered more difficult. For peptides not presenting any miscleavage site, the entire sequence was obtained as observed for *m/z* 1902.78. In all tested cases, derivatization eased protein identification and rendered it more confident. Figure 6 shows the MS/MS spectra of four different peptides issued from on-tissue trypsin digestion, both before and after derivatization with 3-SBASE, and Table 1 summarizes the identification scores obtained with and without derivatization. For all peptides, 3-SBASE derivatization greatly simplified MS/MS spectra and eased sequence determination (Figure 6). An increase in the identification score (Table
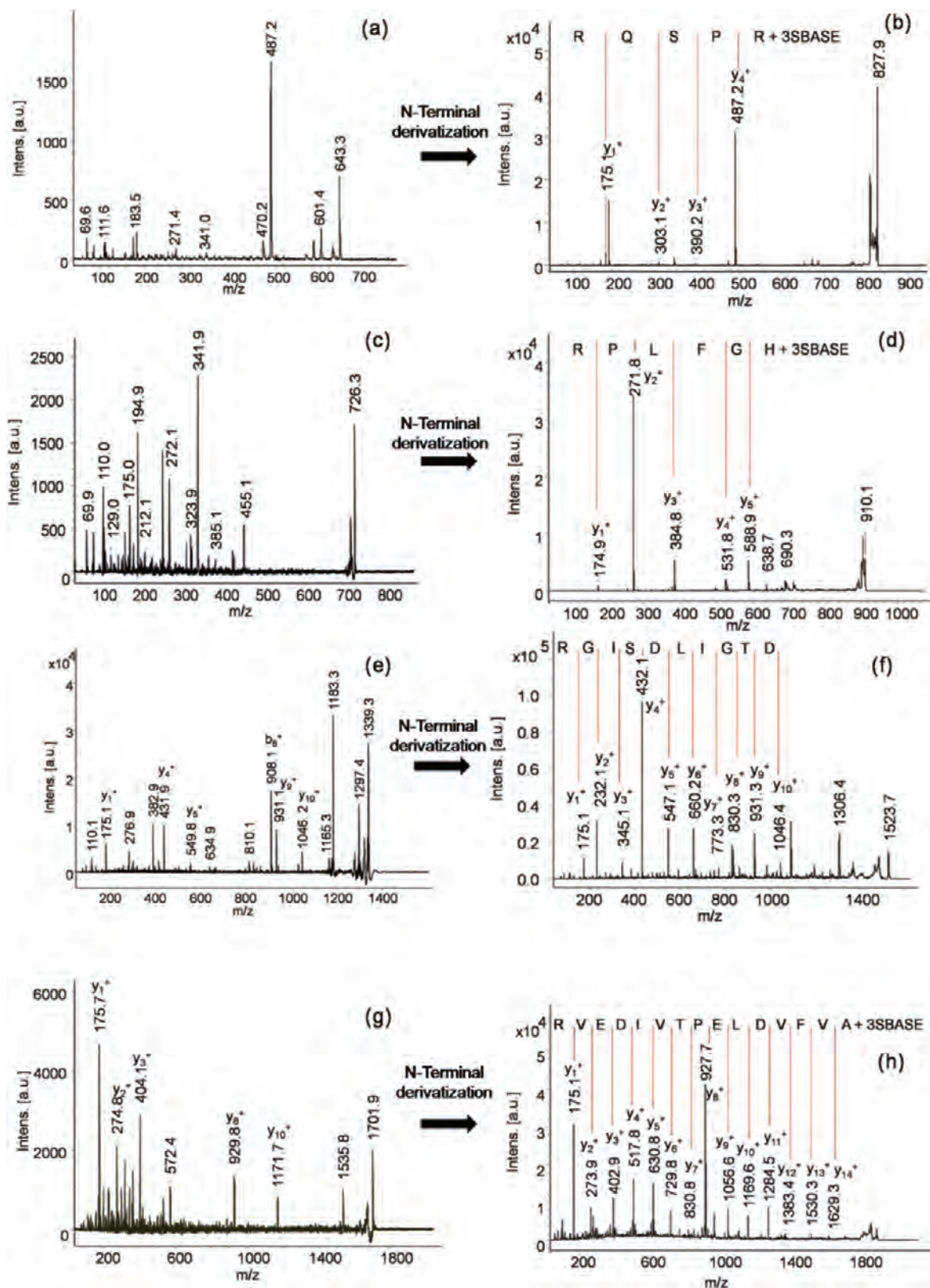
Figure 6. In-tissue MALDI MS/MS spectra recorded after global trypsin digestion (a, c, e, g) or global trypsin digestion followed by derivatization with 3-SBASE (b, d, f, h) of a rat brain tissue section.

1) was clearly observed for all peptides, but the increase was more pronounced for higher mass peptides for which the combination of possible expected m/z (i.e., different fragments) highly increased with the number of residues in the sequence. All of these observations clearly show that derivatization is an interesting approach for helping protein identification directly from tissue sections, especially when integrated in a completely automated sequence.

Table 1. Comparison of Identification Scores as Found from Mascot Query Using the SwissProt Database and *R. norvegicus* Taxonomy for Four Peptides before and after Automated Derivatization with 3-SBASE

	m/z observed	m/z expected	ion score	rank	δ	expect	sequence
peptide	643.35	642.32	NI[a]	NI	NI	NI	RPSQR myelin
peptide + derivatization (3-SBASE)	827.9		46	1	−0.0314	0.0064	basic protein
peptide	726.23	725.22	28	2	−0.1765	0.044	HGFLPR myelin
peptide + derivatization (3-SBASE)	910.1		30	1	−0.3046	0.029	basic protein
peptide	1339.24	1339.71	42	1	−0.4706	0.0015	HRDTGILDSIGR
peptide + derivatization (3-SBASE)	1523.7		93	1	−0.277	9.6×10^{-19}	myelin basic protein
peptide	1701.92	1701.91	57		0.0162	6.2×10^{-5}	AVFVDLEPTVIDEVR
peptide + derivatization (3-SBASE)			142		−0.0058	1.1×10^{-12}	tubulin α-1A chain

[a] NI: not identified.

In-Tissue N-Terminal Derivatization for MALDI Imaging by Automatic Microspotting. In order to combine the valuable in-tissue identification strategy and localization of the identified protein, derivatizations were tested at the microscale level using a microspotting device. In particular, the number of drops per cycle, number of cycles, and time between depositions were studied to optimize the reaction yield. Different buffer solutions were also tested to determine which buffer was most compatible with the piezoelectric deposition. The complete workflow integrated a first step of automated enzymatic digestion (here we used trypsin), followed by an automated derivatization process, and ending with an automated step of matrix deposition. Because 3-SBASE derivatization was shown to be the most efficient derivatization in terms of reaction efficiency (lower reaction temperature, higher yield of derivatization, more peaks detected on tissue) and fragmentation (no loss of the 3-SBASE group as the main fragmentation pathway), this reagent was chosen for automatic derivatization. After automatic trypsin digestion using the microspotter and an incubation period at 37 °C for 1 h, a solution containing 20 mg/mL of 3-SBASE in NH_4HCO_3 buffer (50 mM) was then deposited onto the raster of digestion spots. After 20 iterations, the slide was then incubated (37 °C, 2 h) before matrix deposition. The solid ionic matrix HCCA/ANI was then deposited onto the raster of spots using the same procedure described for tissue digestion. It must be noted that matrix crystallization was difficult or impossible. The spots were then analyzed, but no ion signals were detected. Next, 2,5-DHB was tested; however, similar problems were observed. The concentration of 3-SBASE was then decreased to 10 and 1 mg/mL, respectively, but no N-terminal derivatizations were observed at these concentrations. It was clear that, after derivatization, the ionization efficiency of these peptides was very low in the positive mode. A similar phenomenon was previously observed for these derivatizations in solution.[38] It was shown that the detection of the derivatized peptides were decreased 10-fold in the positive mode due to both the presence of the negative charge of the sulfonic acid group in N-terminal moiety and the presence of salts, which should be considered as impurities that could affect the detection of derivatized peptides. If a decrease in signal intensity is observed after derivatization, intense signals are still observed if sufficient amounts of reagents are used (i.e., micropipet deposition), even when almost no signals are observed by using automated microspotting. Thus, we can assume that the extraction efficiency was lowered for the microspotting due to the small amount of matrix solution deposited. By considering the poor ionization of these derivatized peptides and the low efficiency of extraction using microspotting, it clearly appears that these sulfonation reagents are difficult to introduce in an automatic workflow.

On-tissue derivatization has been shown to be an interesting strategy for confident identification of digestion peptides. Furthermore, 3-SBASE derivatization has shown to be more efficient in terms of reaction yield and fragmentation, though some difficulties can be encountered for peptides containing miscleavages or with the predominance of arginine toward lysine-containing peptides due to higher gas-phase basicity. Other such derivatizations require the presence of a basic amino acid at the N-terminal part of the peptide and must be used in combination with trypsin digestion. For such reasons, derivatization with TMPP was also investigated. Parts d and e of Figure 7 show an example of the resulting MS/MS spectra recorded from a rat brain tissue section after in-tissue trypsin digestion, followed or not by TMPP derivatization. Figure 7d presents the MS^2 spectrum recorded on the tissue section after trypsin digestion. As observed from the MS^2 spectrum presented in Figure 7e, for the underivatized peptide, many fragments of very different types were again present. Most of these fragments were in the low m/z range of the spectrum, and almost no fragments were observed in the upper m/z range, thereby making interpretation difficult. A databank query using this MS^2 spectrum yields an unclear identification. The first protein appearing with the highest identification (score 40, expect 0.0028) was the glial cell line-derived neurotrophic factor for a peptide sequence QAAALPR. The second-ranked protein (28, expect 0.044) was a fragment of the myelin basic protein S, for which the peptide sequence was HGFLPR (Figure 7f). A priori, according to the differences of scores between the two proteins, we could assume our peptide to be a fragment of the glial cell line-derived neurotrophic factor. For identification confirmation, MS^2 was again performed after TMPP derivatization (Figure 7e). As expected for this N-terminal derivatization, the peak corresponding to the peptide of interest was shifted by 573.17 u. After TMPP addition and basic end residue cleavage, the MS^2 spectrum presented a very different fragmentation pattern, with one main fragmentation series corresponding to a_i^+ fragment ions and a few additional weak peaks corresponding to b_i^+ fragment ions. The sequence can easily be manually determined to be HGFLPR. We can therefore confirm that, contrary to what would have been expected from a databank query using the MS^2 spectrum of the underivatized peptide, the peptide was a fragment of the myelin basic protein S.
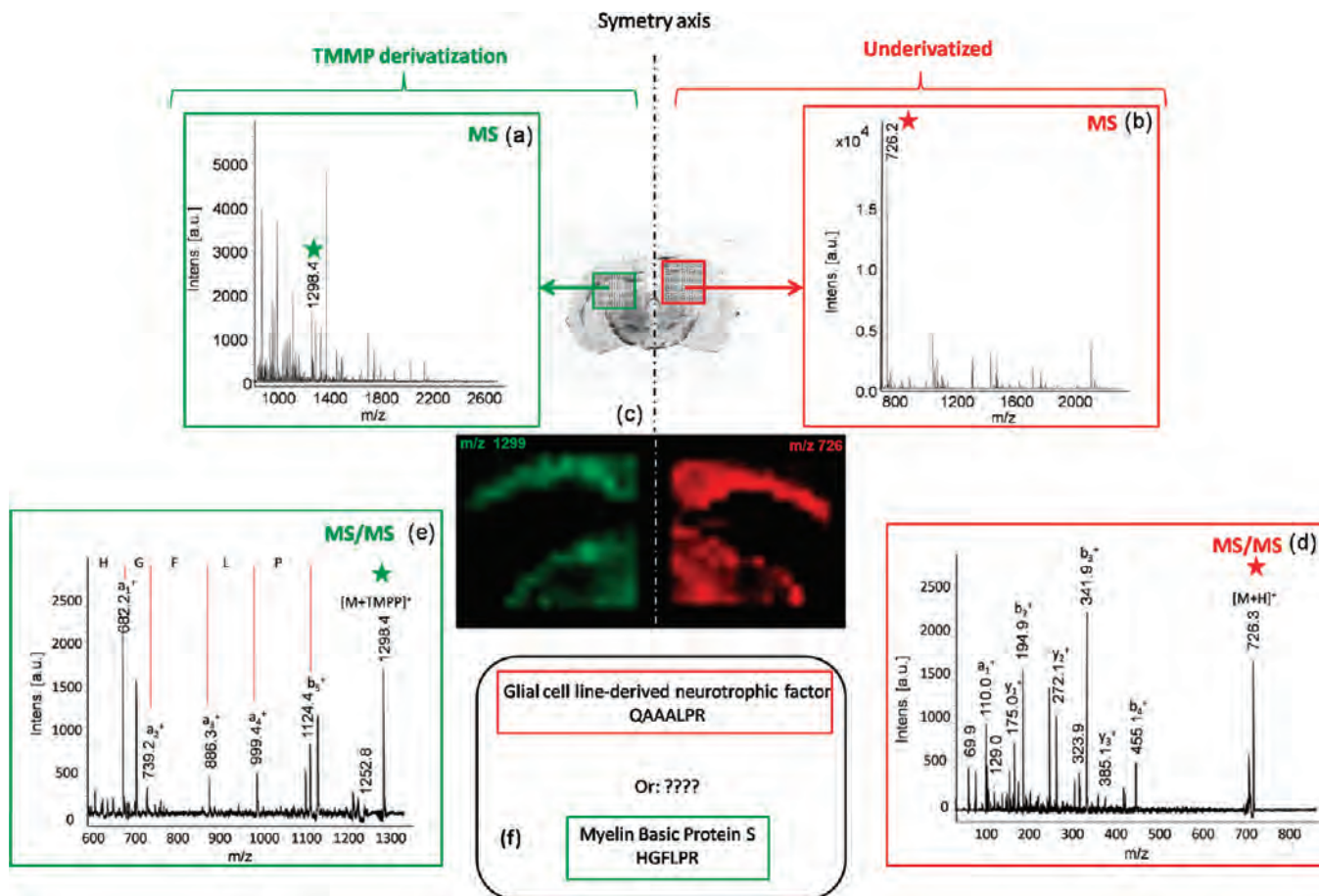
Figure 7. MALDI MS spectra recorded after (a) enzymatic digestion followed by TMPP derivatization and (b) enzymatic digestion. (c) Images of the digestion peptide at *m/z* 726 and its corresponding TMPP derivative at *m/z* 1299, showing an identical distribution for both ions. (d) MALDI MS/MS spectra recorded from a rat brain tissue section in the same area after in-tissue trypsin digestion (e) and in-tissue trypsin digestion followed by TMPP derivatization. (f) Identification results from the databank using *R. norvegicus* taxonomy.

We then tested TMPP to be used in an automated sequence using microspotting deposition. For comparison, after automated digestion of the whole tissue section using microspotting, only half of the brain section (according to the brain symmetry axis) was automatically derivatized by microspotting of the TMPP reagent. This allowed the comparison of the biodistribution of the peptides before and after in-tissue microspotting derivatization by reconstructing molecular images of underivatized and derivatized peptides issued for the tryptic digestion. On the tissue section, two arrays of points were defined. The first, located in the right part of the brain, corresponded to a sequence only involving in-tissue trypsin digestion followed by matrix deposition, whereas the second, located in the left part of the brain, corresponded to a sequence comprising in-tissue trypsin digestion followed by in-tissue TMPP derivatization and matrix deposition. Parts a and b of Figure 7 present the MALDI spectra recorded at the same location on the tissue section according to the symmetry axis of the brain with (Figure 7a) and without (Figure 7b) derivatization. Molecular images of the previously studied peptide identified as a fragment of myelin basic protein S were reconstructed based on the signal of the underivatized peptide (*m/z* 726) and the TMPP-derivatized peptide (*m/z* 1299) (Figure 7c). It clearly appeared that the peptides at *m/z* 726 (underivatized) and at *m/z* 1299 (TMPP-derivatized) presented the same spatial distribution,

illustrating the potential of TMPP for automated in-tissue derivatization and application in MALDI imaging. This derivatization well orients fragmentation toward a_i^+ fragments, but it also resolves the problems of arginine-containing peptide predominance. Derivatization using TMPP does present the advantage of being easier to perform, since the reaction takes place at room temperature to facilitate automation of the strategy. We must also note that problems due to miscleavage sites are no longer observed, since fragmentation is oriented toward N-terminal fragment ions and because charge can no longer be driven by the basic end amino acid. As demonstrated here, TMPP is also a good solution for identification from tissue sections and can be easily used in the imaging mode. We must, however, note that TMPP requires higher fragmentation energy to induce fragment ions. This is due to the fact that fragmentation is not induced by a mobile proton. This can be overcome by increasing the collision energy in instruments equipped with collision cells for CID (some peptides could require 80−90 V).[51] This is not the case for instruments using metastable ion decay, such as PSD or PSD-like modes. In PSD, with respect to the peptide sequence, metastable decompositions driven by an ion's internal energy might not occur on the instrument's time scale,

(51) Chen, W.; Lee, P. J.; Shion, H.; Ellor, N.; Gebler, J. C. *Anal. Chem.* **2007**, *79*, 1583–1590.

leading to MS/MS spectra with very few fragment ions. Thus, for some peptides on MALDI-TOF instruments run in meta-stable decay mode, fragmentations can be difficult to generate. Thus, MALDI-MSI has shown to be possible after in-tissue digestion followed by in-tissue derivatization under automated microspotting conditions of the reagents. Derivatization using TMPP has been shown to be easiest to perform at the microscale level, as the derivatization reaction occurs at room temperature.

DISCUSSION

N-Terminal chemical derivatization of digested peptides has demonstrated to be a valuable strategy for confident and simple protein identification directly from tissue sections. After optimization of derivatization conditions in the tissue, satisfying reaction yield can be obtained and leads to the derivatization of the majority of peptides. Comparison of 4-SPITC and 3-SBASE derivatization has shown that 3-SBASE is more advantageous. 3-SBASE does present higher reaction yields and does not lose the derivative group as one of the main fragmentation pathways. Both 4-SPITC and 3-SBASE derivatization lead to a complete series of y_i^+ fragment ions. In-tissue derivatization can be included in identification strategies directly from tissue sections. We have shown that derivatization can be performed after trypsin digestion to identify peptides, presenting an interesting distribution as determined by MALDI imaging of the peptides resulting from the digestion process. In the identification sequence, derivatization will allow for the confirmation and expedition of the identification of proteins. Derivatizations have been demonstrated to greatly ease identification by databank query or using de novo sequencing approaches, leading to identifications with greater confidence. Despite this, we observed that arginine-terminated peptides were almost the only peptides observed after derivatization. This is due to the strong gas-phase basicity of these peptides compared to the corresponding lysine-terminated ones. In the case of lysine-terminated peptides, a protection using *O*-methyl-isourea[52,53] to convert lysine into homoarginine could be used to increase peptide basicity and thus increase ionization efficiency. This protection would also prevent peptides from multiple derivatizations, which can occur on the amine group found at the end of lysine side chains. We have also observed that for peptides presenting a miscleavage site, the y_i^+ series of ions was stopped, leading to an incomplete sequence. This illustrates the importance of controlling the digestion conditions from tissue sections prior to the N-terminal derivatization. Derivatization with sulfonation agents was automated using automated microspotting of the reagents. Under these conditions, however, derivatization yields were slightly lower compared to larger scale spotting. In particular, problems for the matrix crystallization were observed for derivatizations at the microscale. Varying reagent concentrations have shown to be important parameters in the subsequent crystallization of the matrix. Better crystallization was achieved for lower reagent concentrations, but in such a case, derivatization yields were also affected.

Such effects were not observed in the same conditions for peptide mixtures in solution. This indicates that the observed effects cannot be attributed to the reagent or buffers themselves. Application of the reagent in its buffer on a tissue section without heating the system leads to a normal crystallization of the matrix. This indicates that such problems are coming from the heating of the tissue section in the presence of the reagent.

Derivatizations using TMPP that are independent of the presence of a basic residue at the N-terminal part of peptides (i.e., compatible with different enzymatic digestion) were also studied. Derivatization using TMPP is also usable at the tissue level and allowed for confident identification by orientating the fragmentation toward a major a_i^+ fragment ion series. Derivatization using TMPP was also studied for automated microspotting. This derivatization has shown to be applicable in an automated microspotting sequence on the tissue sections, allowing for image acquisition even after the derivatization process. TMPP was shown to be easier to set up at the microscale level, as the reaction occurs at room temperature. With TMPP, crystallization difficulties in matrix solution were not observed. This is likely related to the reaction temperature (i.e., the room temperature for this reagent). Because TMPP is also independent of the presence and/or position of a basic residue in the peptide sequence, fragmentation orientation is not hampered by the presence of miscleavage in the peptide sequence. Thus, TMPP requires less control of in-tissue digestion; however, TMPP-derivatized peptides require more activation energy for fragmentations to occur. This indicates that, for this derivatization, fragmentation could be difficult for some peptides of specific sequences on instruments involving metastable decay and not CID.

CONCLUSION

On-tissue N-terminal derivatization of in-tissue digested peptides has been shown to be a key point for improved identification of proteins from tissue sections in a MALDI-MSI "bottom-up" strategy. Different derivatizations have been shown to be usable and interesting for in-tissue identification. 3-SBASE and 4-SPITC derivatizations by addition of a negatively charged group at the N-terminus of peptides are useful for peptide identification at the tissue level. 3-SBASE has shown to be more efficient than 4-SPITC. Both derivatization are easily to be performed but must be used in combination with trypsin digestion since they require the presence of a basic amino acid at the C-terminus of peptide. Orientation of fragmentation by derivatization using a positively charged moiety such as TMPP as also proved to be valuable for peptide identification. TMPP presents the advantage to be independent of the presence of basic amino acids at the N-terminus part of the peptides. TMPP derivatization has also shown to be more easily feasible at the microscale level by using automatic microspotting. Inclusion of derivatization procedures in the MALDI-MSI automatic workflow is a good solution for highly confident identification of peptides and proteins directly from tissue sections in MALDI-MSI strategies. This procedure is independent of the conservation of the tissue and can also be used on formalin-fixed paraffin-embedded (FFPE) tissues (archived tissues), if required, since FFPE tissue must be submitted to digestion procedures for MALDI-MSI. This will pave the way for

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(53) Beardsley, R. L.; Reilly, J. P. *Anal. Chem.* **2002**, *74*, 1884–1890.

in-tissue identification of pathological markers directly from archived materials from the hospital.

ACKNOWLEDGMENT

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