

# Histology-Directed Microwave Assisted Enzymatic Protein Digestion for MALDI MS Analysis of Mammalian Tissue

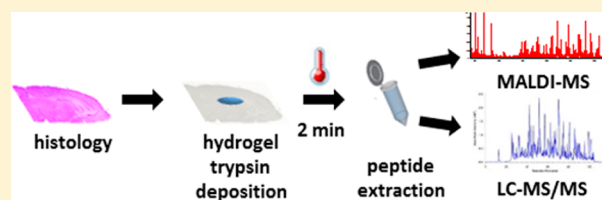
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## S Supporting Information

**ABSTRACT:** This study presents on-tissue proteolytic digestion using a microwave irradiation and peptide extraction method for *in situ* analysis of proteins from spatially defined regions of a tissue section. The methodology utilizes hydrogel discs (1 mm diameter) embedded with trypsin solution. The enzyme-laced hydrogel discs are applied to a tissue section, directing enzymatic digestion to a spatially confined area of the tissue. By applying microwave radiation, protein digestion is performed in 2 min on-tissue, and the extracted peptides are then analyzed by matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS). The reliability and reproducibility of the microwave assisted hydrogel mediated on-tissue digestion is demonstrated by the comparison with other on-tissue digestion strategies, including comparisons with conventional heating and in-solution digestion. LC-MS/MS data were evaluated considering the number of identified proteins as well as the number of protein groups and distinct peptides. The results of this study demonstrate that rapid and reliable protein digestion can be performed on a single thin tissue section while preserving the relationship between the molecular information obtained and the tissue architecture, and the resulting peptides can be extracted in sufficient abundance to permit analysis using LC-MS/MS. This approach will be most useful for samples that have limited availability but are needed for multiple analyses, especially for the correlation of proteomics data with histology and immunohistochemistry.



Tissue analyses, including histomorphological and immunohistochemical approaches, form the basis for most diagnostic analyses in anatomic pathology.<sup>1</sup> Highly standardized approaches and rigorous training regimens have been instituted to ensure that these morphological approaches to disease characterization deliver a high standard of care. However, there still exist situations for which the current methods do not provide definitive diagnoses and new technological approaches that incorporate molecular analysis would add significant value to the diagnostic process.<sup>2</sup> The development of proteomics and mass spectrometry technologies during the previous decade has enabled rapid and specific protein analyses. These technical advances now provide the opportunity to contribute molecular information with high chemical and spatial specificity at sufficient throughput to aid in the histopathological evaluation of patient specimens.<sup>3–8</sup>

Protein analysis and identification are traditionally performed through the use of one of two different strategies. Proteins can be separated by gel electrophoresis in one or two dimensions (1D/2D) and enzymatic digestion is performed in-gel, a time-consuming and manual process.<sup>9</sup> In a second solution-based approach, proteins or peptides can be separated chromatographically using on-line liquid chromatography (LC) systems and the proteins digested in solution prior to the chromatographic analysis.<sup>10</sup> The in-solution approach tends to be the simplest in terms of sample handling and speed, but the

digestion step is still the most time-consuming step in the sample preparation workflow.<sup>11,12</sup> Another disadvantage to this approach is the requirement for sample homogenization. Common proteomics workflows such as those described require microgram to milligram quantities of proteins to be extracted from the tissue to provide sufficient material to perform the analysis. This requires the homogenization of the bulk sample, a step that can significantly diminish the possibility of studying specific groups of cells in relation to their native environment in the tissue.

Histology-guided approaches for the analysis of tissues have been developed that can overcome these problems. For example, many groups have reported the use of laser microdissection (LM) to sample specific cell types from tissues (both fresh and formalin fixed)<sup>13,14</sup> and subsequently analyze these samples using a variety of genomics and proteomics approaches.<sup>15–17</sup> This approach has been utilized to study the molecular content in histologically distinct tissue regions in a variety of disease states.<sup>18–20</sup> Furthermore, there now exists a proteomics-based diagnostic test that combine LM with liquid chromatography tandem mass spectrometry (LC-MS/MS) to type specific amyloid proteins in patient biopsies.<sup>21</sup> In spite of

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the advantages and the utility of LM as a sampling approach for proteomics of tissue specimens, throughput is very limited, making it difficult to be used routinely.

In a new approach, digestion is performed directly on cryosectioned tissue, and the constituent peptides of the proteins contained therein are identified directly from the tissue by tandem MS (MS/MS) and accurate mass measurements. The bottom-up approach, including *in situ* proteolytic digestion, is often used to identify a pool of proteins from which many potential biomarkers are most likely derived.<sup>22</sup>

Many traditional proteomic methodologies to identify proteins may involve one of several steps such as micro-extraction with solvents from the tissue surface, tissue homogenization using multiple tissue sections or LM of the regions of interest in a single tissue section.<sup>23–25</sup> All of these approaches require overnight digestion, a procedure that can be problematic for analyses of such small volumes on-tissue surfaces where evaporation and delocalization of the solvent can stop the digestion prematurely.

The enzymatic digestion step is commonly the bottleneck of the workflows used in proteomics. Previously, many research teams have developed new protocols for protein digestion and identification that are designed to reduce the sample handling while increasing sample throughput.<sup>26</sup> These two goals have been achieved by reducing the total time of the entire workflow or increasing the number of samples treated at the same time.<sup>10</sup> Many tools have been successfully used to accelerate the enzymatic digestion of proteins: for example, heating, micro-spin columns, ultrasonic energy, high pressure, infrared energy, alternating electric field or microwave.<sup>9,27–30</sup> While microwave assisted proteolytic digestion has traditionally been implemented in solution, there is a growing trend to use heterogeneous systems for on-tissue digestion in which enzyme is carried within hydrogels or adsorbed on solid supports.<sup>31,32</sup>

Molecular hydrogels have attracted extensive research interest because of their great potential for tissue engineering, migration of organic and inorganic material, drug delivery as well as a miniaturized method for application on biological samples.<sup>32–36</sup> Recently, Harris et al. performed on-tissue digestion within an ionotropic hydrogel on fresh frozen rat brain tissue utilizing chromatography paper as a fabrication template.<sup>37</sup> Further, Nicklay et al. have used these hydrogel devices for the identification of integral membrane proteins, optimizing a specific tissue washing step.<sup>38</sup>

In the current work, we combine the use of hydrogel disks (1 mm diameter) embedded with trypsin solution and the use of microwave irradiation (2 min) to speed the enzymatic digestion for protein identification while sampling a histologically defined region on the tissue. The objective of this study was to develop an on-tissue microwave assisted protein digestion method that optimizes the sample handling and increases the protein identification efficiency for LC-MS/MS analysis. More peptides and higher sequence coverage could be obtained from the proteins extracted by the hydrogel/microwave digestion method as compared to digestion approaches that utilize conventional methods of extraction and heating. We illustrate the performance of this method for histology-directed on-tissue digestion for the proteome analysis of rat brain tissue, targeting the thalamic region.

## MATERIALS AND METHODS

Acrylamide/bisacrylamide was purchased from Biorad Life Sciences (Hercules, CA, USA). Ammonium persulfate,

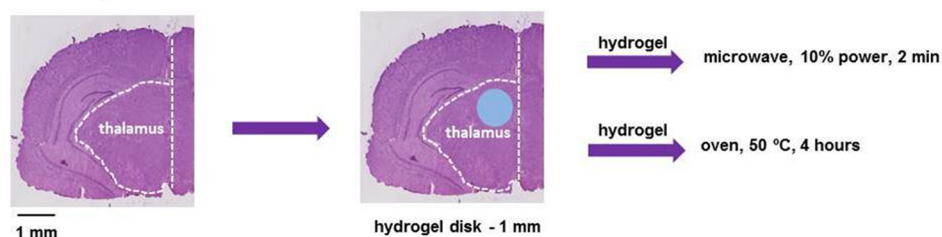
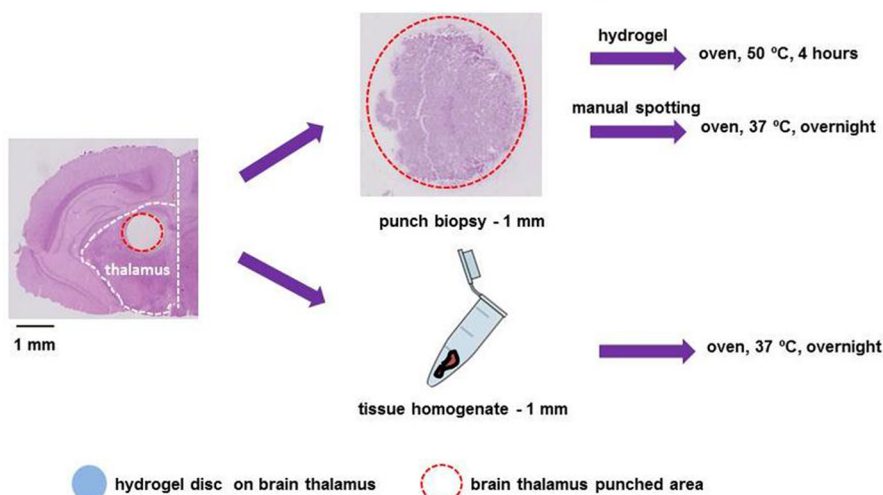
ammonium bicarbonate, the MALDI matrix  $\alpha$ -cyano-4-hydroxycinnamic acid (98%), trifluoroacetic (TFA) and formic (FA) acids were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Punch biopsies were purchased from Acuderm, Inc. (Ft. Lauderdale, FL). Mass spectrometry grade Trypsin Gold was purchased from Promega Corporation (Madison, WI, USA), high performance liquid chromatography (HPLC) grade solvents (ethanol, xylenes, methanol and acetonitrile) and histological dyes (hematoxylin and eosin) were from Fisher Scientific (Fairlawn, NJ, USA), and xylene was from Acros (Morris Plains, NJ). Water was generated using a Millipore Milli-Q Synthesis A10 (Billerica, MA, USA). All reagents listed were used without additional purification.

**Hydrogel Discs Fabrication.** Fabrication of 7.5% poly-acrylamide hydrogels was carried out by modification of a previously developed procedure.<sup>38</sup> A volume of 1.24 mL of 30% acrylamide/bis(acrylamide) solution was added to 1.26 mL of Tris buffer at pH 6.8 and 2.45 mL of water. The solution was degassed under vacuum for a minimum of 30 min before adding 50  $\mu$ L of 10% ammonium persulfate and of 10  $\mu$ L of tetramethylethylenediamine (TEMED). The solution was mixed by inversion and placed into a small Petri dish to polymerize for 30 min. Finally, punch biopsy tools were used to cut the microwells in a variety of sizes (1, 1.5, 3 mm diameter). Each individual microwell of hydrogel was placed in an eppendorf tube, dried fully in a speedvac and stored at  $-80^{\circ}\text{C}$  until use.

**Tissue Sectioning and Pretreatment.** Fresh frozen rat brain was purchased from Pel-Freez Biologicals (Rogers, AZ) and tissue sections were prepared at an 8  $\mu\text{m}$  thickness using a Leica CM3050 cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Frozen tissue sections were thaw mounted on microscope slides or placed into eppendorf tubes (for the homogenization procedure) and stored in a desiccator until needed. Each tissue section was rinsed using ethanol (70%, 30 s; 95%, 30 s) to remove salts and lipids and to obtain optimal sensitivity for MS analysis of the digested extracts.<sup>39</sup>

**On-Tissue Microwave Digestion.** Hydrogels (1 mm diameter) were rehydrated for 15 min using 20  $\mu$ L of 1  $\mu\text{g}/\text{mL}$  trypsin (in 100 mM ammonium bicarbonate) and then placed over the tissue region of interest (brain thalamic region) onto the whole tissue surface guided by the histological features on corresponding serial H&E stained tissue section. The tissue sections were incubated in a microwave oven (1.65 kW) for 2 min set at 10% of the power, to accelerate protein digestion. Each hydrogel disc was removed from the tissue section and placed in separate eppendorf tubes. Peptides imbibed into the microwell hydrogels were extracted by organic (50% acetonitrile/5% formic acid) and aqueous (100 mM ammonium bicarbonate) solvents, a process that was repeated three times. The supernatants collected from each extraction were combined and dried in a centrifugal vacuum concentrator (SPD Speedvac, Thermo Scientific, Waltham, MA, USA). The reconstituted extracts (20  $\mu$ L, 0.1% formic acid) were spotted for matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) analysis, mixed with CHCA, 10 mg/mL in 50% acetonitrile, 0.5% TFA, and then stored at  $-20^{\circ}\text{C}$  until LC-MS/MS analysis was performed.

**Other On-Tissue Digestion Strategies.** Further protein digestion experiments were carried out on serially prepared tissue sections. First, tissue sections were incubated using a conventional oven at  $50^{\circ}\text{C}$  for 4 h: hydrogel discs were still used to allow the digestion to take place on the brain thalamic

a) rat brain – 8  $\mu\text{m}$  sections of whole tissueb) rat brain – 8  $\mu\text{m}$  sections from 1mm diameter punch biopsy

**Figure 1.** Workflow for histology-directed on-tissue enzymatic digestion. (a) H&E of a fresh frozen rat brain tissue section (cryosectioned at 8  $\mu\text{m}$ ), stained for histological evaluation and localization of the brain thalamic region. Enzymatic digestions were performed depositing the hydrogel disc embedded with trypsin on the thalamic region and then incubating the tissue section into the microwave for 2 min; further, a consecutive cut tissue section was incubated in an oven. (b) Rat brain punch biopsy was obtained from the thalamic region at the same diameter of the hydrogel disc (1 mm) and then cryosectioned at 8  $\mu\text{m}$ . Protein digestion experiments were carried out using the hydrogel device as well as manual spotting the enzyme solution and homogenizing tissue sections within a conventional oven.

region. After digestion, peptides were extracted from the gel following the same procedure already described for the microwave digestion. Second, because hydrogels were fabricated at 1 mm diameter, the rat brain biopsy was also punched into the thalamic region using a 1 mm punch biopsy tool. This approach precisely controls the amount of tissue exposed to the hydrogel and allows for the homogenization and digestion of the same amount of tissue using conventional sample preparation methods. Serial sections from the 1 mm tissue core were cryosectioned following the same protocol described above. One set of digestion experiments was carried out:  $n = 3$  tissue core sections were mounted on microscope slides and hydrogel discs (trypsin embedded) were placed on top and incubated via conventional oven at 50 °C for 4 h. Second,  $n = 3$  tissue core sections were marked using a hydrophobic pen and trypsin (20  $\mu\text{L}$  of 1  $\mu\text{g}/\text{mL}$  trypsin in 100 mM ammonium bicarbonate) was manually spotted and incubated overnight at 37 °C. Finally, other serial tissue core sections from the same tissue specimen were placed into separate eppendorf tubes and the homogenized as previously described.<sup>40</sup> The digestion was conducted at 37 °C overnight, and the digested peptides were extracted following the same procedure described for the hydrogel experiments.

**Mass Spectrometry Analysis and Data Processing.** MALDI MS analyses were carried out using an Ultraflextreme

MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a SmartBeam II laser and operating in positive polarity, reflectron mode. Spectra were acquired in the range of  $m/z$  500–4000. Flex Control 3.3 software was used for spectra acquisition. The reproducibility of the hydrogel-based digestion was evaluated using a set of three technical replicates within the same tissue sample at different but histologically identical locations. All spectra were processed using the same preprocessing procedure to ensure overall consistency. Briefly, they were baseline-corrected and normalized according to their total ion current, excluding the top 5% of intensity values to avoid bias by highly abundant species. The Mann–Whitney U test and Kruskal–Wallis test were applied to evaluate statistically significant differences (protein groups and distinct peptides) between groups (all different digestion strategies). The Mann–Whitney U test and Kruskal–Wallis test are the analogous nonparametric methods of  $t$ -test and one-way between-groups of variance (analysis of variance, ANOVA), respectively.

**LC-MS/MS analysis.** Tryptic peptides were analyzed by a 70 min data dependent LC-MS/MS analysis. Briefly, peptides were loaded via pressure cell onto a 40  $\times$  0.1 mm self-packed reversed phase (Jupiter 5  $\mu\text{m}$ , 300 Å - Phenomenex) trapping column fritted into an M520 filter union (IDEX). After loading and equilibration, this trapping column was attached to a 200  $\times$

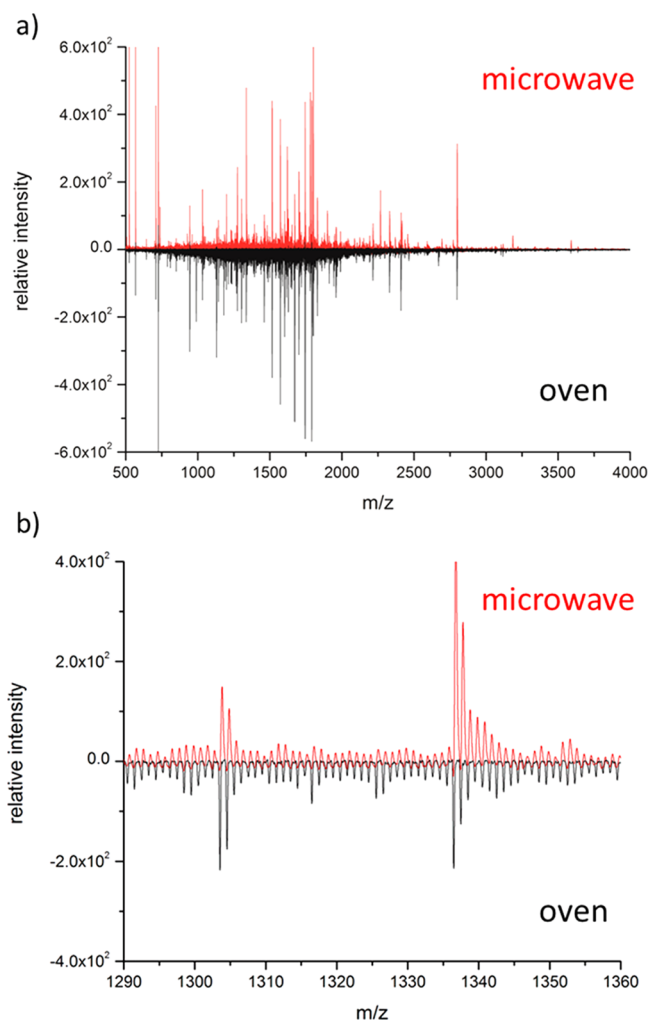


0.1 mm (Jupiter 3  $\mu\text{m}$ , 300A), self-packed analytical column coupled directly to an LTQ (ThermoFisher) using a nano-electrospray source. A series of full scan mass spectra followed by 5 data-dependent tandem mass spectra (MS/MS) was collected throughout the run, and dynamic exclusion was enabled to minimize acquisition of redundant spectra. MS/MS spectra were searched to identify tryptic fragments via SEQUEST against a rat database (UniprotKB, *Rattus norvegicus*) that also contained a reversed version for each of the entries.<sup>41</sup> Identifications were filtered to 2 peptides per protein and 0.1% peptide false detection rate and collated at the protein level using Scaffold (Proteome Software). Furthermore, IDPicker 3 software used to filter the resulting identifications to a 5% FDR at the peptide level and collate the individual proteins, requiring a minimum of 2 peptides per protein.<sup>42</sup>

## RESULTS AND DISCUSSION

The aims of this study were to demonstrate both the reliability and the relative advantages of the use of microwave radiation to speed up the on-tissue proteomics workflow and to demonstrate the methodology to perform the enzymatic digestion in a histologically defined region on a thin 8  $\mu\text{m}$  tissue section. Various on-tissue digestion strategies were carried out on the same sample to provide a basis for comparison. A series of experiments were designed and carried out using rat brain serial sections from the same tissue specimen, to avoid tissue proteome variability. Figure 1 shows a graphical depiction of the experimental design used in this study. Fresh frozen rat brain biopsies were first sectioned at an 8  $\mu\text{m}$  thickness and then stained by hematoxylin and eosin (H&E) for histological evaluation of regions of interest, such as the thalamic region. Hydrogel discs, prepared on a prior day and stored, were reconstituted in trypsin solution for 15 min and then placed on the rat brain thalamic region. First, on-tissue digestion experiments were carried out: Figure 1a shows two different digestion strategies (microwave and conventional heating) performed on the whole tissue section using the hydrogel disc for the histology-directed digestion. Figure 1b displays another set of experiments performed on the rat brain thalamic region punched from the bulk biopsy specimen from a prior cryosection. The bulk specimen was stained to provide a visual comparison of the areas sampled for analysis. The biopsied tissue from the bulk specimen was sectioned at a thickness of 8  $\mu\text{m}$  to collect an amount of tissue comparable for that of the on-tissue digestion.

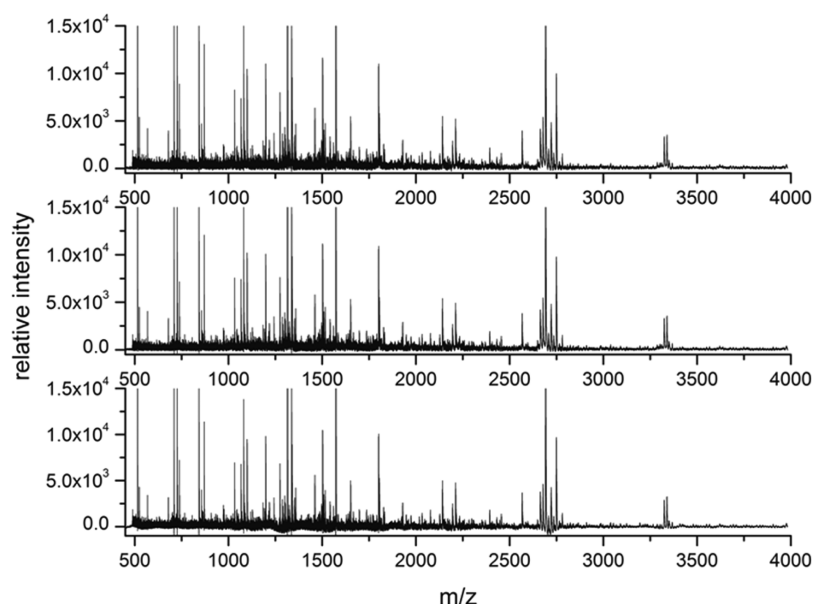
Solvent extracted digested peptides obtained from the first two hydrogel experiments, carried out on-tissue via microwave and oven, were reconstituted and mixed with a solution of CHCA for MALDI MS analyses. The resulting profile MALDI spectra are displayed in Figure 2. The peptide spectra are qualitatively comparable, with most major ions present in both preparations in the mass range measured (500–4000 Da). While most peaks are present, there are notable relative intensity differences, likely due to the different incubation strategy (microwave vs conventional oven). This result should have little impact on downstream identification by LC-MS/MS; however, these differences would not permit quantitative comparisons between samples that have been prepared using two different on-tissue digestion approaches. MALDI MS profiles of three technically replicated microwave assisted hydrogel mediated on-tissue digested extracts are presented in Figure 3. Most of the signals are detected in all the three



**Figure 2.** MALDI MS spectra of the solvent extracted digested peptides obtained from the first two hydrogel experiments, carried out via microwave (red) and oven (black). The resulting profiles display a high degree similarity in the ions present in the mass range 500–4000 Da (a) and in the mass range 1290–1360 Da (b).

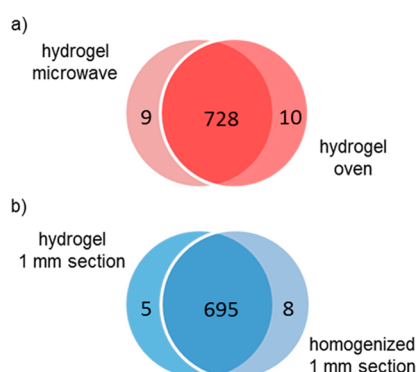
replicates, confirming the reliability of the microwave procedure for the digestion.

To further validate the on-tissue hydrogel/microwave digestion approach, other on-tissue digestion experiments were carried out as described in Figure 1b. Serial sections at 8  $\mu\text{m}$  thickness and 1 mm diameter were cut from the thalamic region of rat brain as described in the Materials and Methods section. This experiment was performed to further validate the extraction localized proteins by the hydrogel discs through the exposure of a tissue surface cut at the same diameter which the hydrogel discs were fabricated (1 mm). Rat brain thalamus proteins were digested following three approaches: (1) using a hydrogel disc and incubating the reaction for 4 h at 50  $^{\circ}\text{C}$ , (2) by deposition of the trypsin solution onto the tissue surface and (3) by the homogenization of tissue sections followed by protein extraction (see the Materials and Methods section); both the last two approaches were allowed for digestion by overnight incubation at 37  $^{\circ}\text{C}$  (Figure 1b). All the digested extracts, were analyzed by LC-MS/MS followed by database search for protein identification. Data were processed using a 5% FDR, filtering the identified proteins with number of unique



**Figure 3.** MALDI MS spectra of the three technical replicates of microwave assisted hydrogel mediated on-tissue digested extracts. The resulting profiles display a high degree similarity in both the ions present and their relative abundance in the mass range 500–4000 Da.

peptides  $\geq 2$  and a  $p$ -value  $< 0.05$ . Data are summarized in Figure 4 using Venn diagrams.

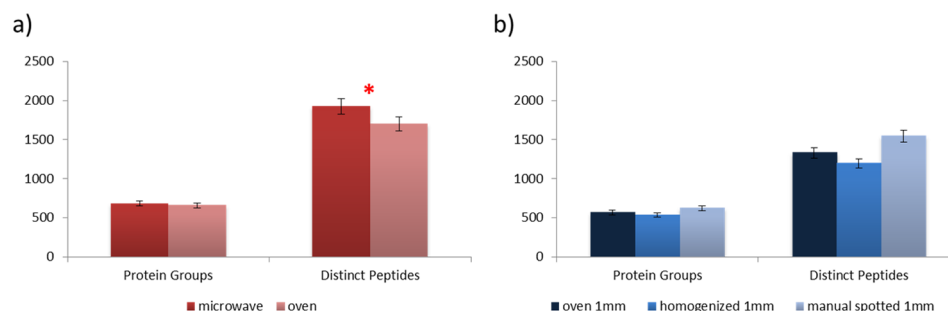


**Figure 4.** Venn diagrams summarizing the number of identified proteins for  $N = 3$  on-tissue digestion experiments (5% FDR,  $\geq 2$  unique peptides and  $p$  value  $< 0.05$ ). Panel a shows the number of identified proteins within the microwave digested extracts and the oven incubation digested extracts, both carried out using the hydrogel disc placed onto the rat brain thalamic region; panel b displays the number of identified proteins within the experiments performed using the 1 mm diameter tissue section from the same region of interest within the rat brain biopsy, using the hydrogel disc and homogenizing the tissue sections, respectively.

The digestion strategies were evaluated and compared in different ways: the first comparison was carried out considering the number of identified proteins found when using microwave heating compared to conventional heating in an oven. A large number of proteins ( $\sim 700$ ) were identified in both the microwave digestion strategy and the oven incubation, both using the hydrogel disc (Figure 4a); this finding suggests that the hydrogel disc device allows for a comparable degree of digestion in 2 min using microwave heating as well as in 4 h using conventional heating. Relatively few proteins were uniquely identified in one or the other approaches, respectively 9 from the microwave digestion and 10 from the oven

digestion. This finding confirms that by changing the method of heating to microwave irradiation, we do not significantly alter the protein population sampled from the brain tissue. Moreover, Figure 4b displays a Venn diagram comparing the number of identified proteins within the experiments performed using the 1 mm diameter tissue sections from the thalamic region of the rat brain. Also in this case, the majority of the identified proteins (695) were identified using both digested extracts (from the hydrogel disc and from the tissue homogenization), while a few proteins were found uniquely expressed into the two set of samples, respectively 5 from the sample from the hydrogel digestion and 8 from the homogenization process (Figure 4b). Because homogenization is considered to be the most comprehensive method of protein extraction from tissue, allowing for the most complete disruption of tissue and cell architecture among the methods tested, the hydrogel disc method displays a remarkable similarity to the results obtained using the conventional homogenization approach. The manual spotted digestion extracts were evaluated: the number of identified proteins was found similar to the number of identified proteins from the other strategies considered in this study (Figure S-2, Supporting Information).

All the different on-tissue digestion strategies were further validated using the LC-MS/MS data from the replicated experiments. Thus, two parameters were considered for the comparison. The first parameter was the number of protein groups, which defines the minimum number of uniquely identified proteins (when several possible proteins have highly similar sequences and cannot be distinguished by the peptides identified in a given experiment, these proteins are reported as a single group). The second parameter used for the evaluation of all the different digestion strategies was the number of distinct peptides: peptides are considered distinct when they identify unique sequences of amino acids. Figure 5a displays the number of protein groups and distinct peptides that were obtained from the hydrogel digestion experiments performed using both the microwave for 2 min and the conventional oven for 4 h: results were found to be very similar for all the metrics



**Figure 5.** Number of protein groups and of distinct peptides obtained from the hydrogel experiments carried out via microwave and oven (a) and from the experiments performed on the 1 mm diameter rat brain tissue sections from thalamic region (b). The results are expressed as mean  $\pm$  SD ( $N = 3$ ). Data are averaged from  $N = 3$  replicated experiments per class. An asterisk denotes statistically significance among the comparison performed.

compared. These results, along with those of Figure 4a confirm that accelerating the on-tissue digestion using microwave radiation is reliable and it allows for an almost identical population of proteins to be identified. Moreover, very few differences were found when comparing the number of distinct peptides; however, we observed a statistically significant increase of the peptides identified in the microwave digestion experiments. Figure 5b illustrates the comparison between the experiments performed on the 1 mm diameter rat brain thalamic region. In this case, the number of protein groups was not significantly changed among all the digestion strategies (Figure 5b). When the number of distinct peptides was considered, the manual spotting digestion strategy gave higher values, although the digestion efficiency of these approaches may have differed. However, given that the same amount of trypsin was used in all the methods, using the hydrogel disc as well as in the classic on-tissue digestions, the difference may be due to the limited volume of the hydrogel and also to the different incubation step (microwave vs oven). Furthermore, the Mann–Whitney U test (for the hydrogel digestion experiments performed via microwave and oven) and Kruskal–Wallis test (for all the digestions carried out using the 1 mm rat brain tissue sections) were applied to the number of protein groups and the number of distinct peptides in order to find possible statistical differences between groups. Significant difference was found only for the number of distinct peptides within the comparison microwave/oven ( $p < 0.05$ ).

## CONCLUSIONS

We have developed a method to significantly speed up on-tissue protein digestion by applying microwave irradiation for 2 min. This method was demonstrated to provide histology-directed analysis at resolutions down to 1 mm diameter that are precisely placed on defined regions, localizing the digestion to a defined area of the tissue. The reliability and reproducibility of the microwave assisted digestion has been demonstrated by the comparison of the number of identified proteins and other data from the LC-MS/MS experiments. This study demonstrates that a rapid and reliable protein identification strategy can be performed on a single tissue section while preserving the inherent spatial information on the tissue. This is of primary importance when the amount of material (tissue biopsy) is often not enough for proteomics as well as for all the other analysis that are usually carried out on a biopsy for clinical investigations. In contrast, the conventional tissue homogenization and digestion procedures are slower, more time-consuming and have a significantly higher number of steps.

This often results in the need for a higher amount of starting material because of sample loss resulting from handling of the tissue. Moving forward, the hydrogel discs fabrication can be optimized for different dimensions, according to tissue regions of interest. Future work will also include optimization of the hydrogel methodology for multiple enzymes experiment as well as for intact protein analyses. Furthermore, we will continue the development of fabrication methods to further the use of hydrogel mediated digestion to smaller tissue areas. Taken together, these results suggest the possible clinical utility of histology-directed protein digestion approach.

## ASSOCIATED CONTENT

### Supporting Information

Workflow of hydrogel discs fabrication and of the on-tissue digestion, Venn diagrams summarizing the number of identified proteins for  $N = 3$  on-tissue digestion experiments, summary chart of the LC-MS/MS data from all the on-tissue protein digestion experiments carried out in this study, reproducibility of the LC-MS/MS data from the hydrogel mediated on-tissue digestion experiments carried out on the rat brain thalamic region, and reproducibility of the LC-MS/MS data for the on-tissue digestion experiments carried out on the 1 mm thalamic region punched from the bulk rat brain biopsy. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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