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David Touboul
Natural Product Chemistry Institute
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French National Centre for Scientific Resea…
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Letter to the Editor

To the Editor-in-Chief
Sir,

Characterization of alpha-parvalbumin on muscle tissue sections by *in situ* calcium attachment

Many proteins recruit metal atoms as intrinsic parts of their structures. Metal ions participate in a variety of functions in proteins, the most important of them being the enhancement of the structural stability of the protein in the conformation required for biological function, and/or to take part in the catalytic processes of enzymes.[1] Mass spectrometry (MS), and especially electrospray ionization (ESI),[2] has been widely used to study the stoichiometry and the binding constants of metals to proteins.[3–5] Nevertheless, this technique requires purified samples and hence long steps of extraction and separation prior to analysis. Moreover, several factors can affect the transfer of the protein/metal complex into the gas phase, e.g. the buffer added to the solvent and the pH value of the electrosprayed solution, or the gas pressure in the interface region.[6]

Matrix-assisted laser desorption/ionization (MALDI) is known to be more tolerant to salts and impurities than ESI.[7] Numerous studies have reported the observation of non-covalent complexes by MALDI-MS although difficulties during sample preparation restrict the use of this method.[8] Ten years ago, Caprioli and co-workers showed the ability of MALDI-MS to obtain protein ions directly from tissue sections.[9] This technique, known as protein profiling, can be used to differentiate at the molecular level different areas of the biological tissue under investigation.[10] In such studies, only some solvent washes are necessary and the matrix can be easily deposited as individual droplets. This easy experimental protocol prompted us to try this approach to study the metal affinity of parvalbumin α, which is an abundant protein in muscular tissues, by direct MALDI-MS analysis of thin-layer muscle sections.

Acetonitrile, sinapinic acid (SA) and metal salts ($AlCl_3$, $CrCl_3$, $NiCl_2$, $ZnCl_2$, $FeCl_2$, $CoCl_3$, $MnCl_3$, $CdCl_3$, $CaCl_2$, CsCl) were purchased from Sigma–Aldrich (St Quentin Fallavier, France). The ProMix1 protein mixture (Laser-Bio Labs, Sophia-Antipolis, France), which contains bovine insulin (m/z 5734.6), horse heart cytochrome C (m/z 12361.1) and horse myoglobin (m/z 16952.5), was used for calibration of the MALDI positive ion mass spectra. All the matrix solutions were prepared by mixing (10:1, v/v) the SA stock solution (30 mg · mL^{-1} in water/acetonitrile, 1:1, v/v) with the metal salt solution (100 mM in water/acetonitrile, 1:1, v/v). The concentration of the metal salt solution was optimized in order to achieve good sensitivity and reproducibility. Mouse leg muscles were cooled to −160°C in liquid nitrogen after dissection and stored at −80°C. They were cut at a temperature of −20°C, and to a thickness of 20 μm, using a LEICA CM3050 S cryostat (Leica Microsystems SA, Rueil-Malmaison, France). The tissue sections were deposited on conductive glass slides (Delta Technologies, Stillwater, MN, USA) and dried under a pressure of a few hPa for 15 min just before the analysis. Volumes of 500 nL of each matrix/metal solution were then successively deposited three times at different points of the tissue sections, leading to a final matrix spot of about 1 mm diameter. The data were recorded on a Voyager DE-STR (Applied Biosystems, Les Ulis, France) time-of-flight mass spectrometer equipped with a delayed-extraction ion source. Positive ion mass spectra were acquired in the linear mode, by accumulating about 1000 shots per matrix spot. Under the current laser fluence, the diameter of the area ablated by the laser beam was around 50 μm. External mass calibration was performed by using the ProMix1 solution. Correction of the baseline, noise filtration, and five-point smoothing were applied to each spectrum.

A control mass spectrum obtained on a mouse leg muscle tissue section, with a SA matrix solution and without any metal salt addition, is reported in Fig. 1. Signals of the α- and β-hemoglobin chains, thymosin β4 and ubiquitin were easily detected. Their identification was made on the basis of many previous studies carried out on various tissue types.[11] Using these peaks, the mass calibration was refined, leading to the identification of two other major proteins, myoglobin (average m/z 16940, P04247 in Swiss-Prot Data Base[12]) and *N*-acetylated parvalbumin α (average m/z 11837, P32848 in Swiss-Prot Data Base). Parvalbumin α, a calcium-binding protein, is involved in muscle relaxation and myoglobin serves as a reserve supply of oxygen. Both proteins are known to be mainly located in muscles.

Figure 2 displays the partial mass spectra obtained from mouse muscle tissue sections by the use of matrix solutions containing various metal salts. With Al^{3+}, Cr^{3+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+} and Mn^{2+} ions (Figs. 2(a)–2(g)), only a Poisson-like distribution of the peaks corresponding to the parvalbumin/metal complexes was observed, with 1 to 4 metal cation(s) attached on the protein molecule. This result can be rationalized by the formation of non-specific adducts, due to the high salt concentrations.[13,14] The number of metal cations in these non-specific adducts could be correlated neither to the charge states of the metal ions, nor to their ionic radii (Table 1). In the case of Cd^{2+} and Ca^{2+} (Figs. 2(h) and 2(i)), the peak patterns were significantly different. The major peak corresponded to [Parvalbumin+2Metal-3H]$^+$, indicating specific interactions or effects related to the larger ionic radii of these two cations. This second hypothesis was ruled out by using a CsCl salt (Fig. 2(j), which led only to non-specific interactions. Further, all the other protein peaks showed only non-specific interactions with all the salts used. Consequently, two specific binding sites of calcium/cadmium were highlighted on parvalbumin α by direct analysis of mouse muscular

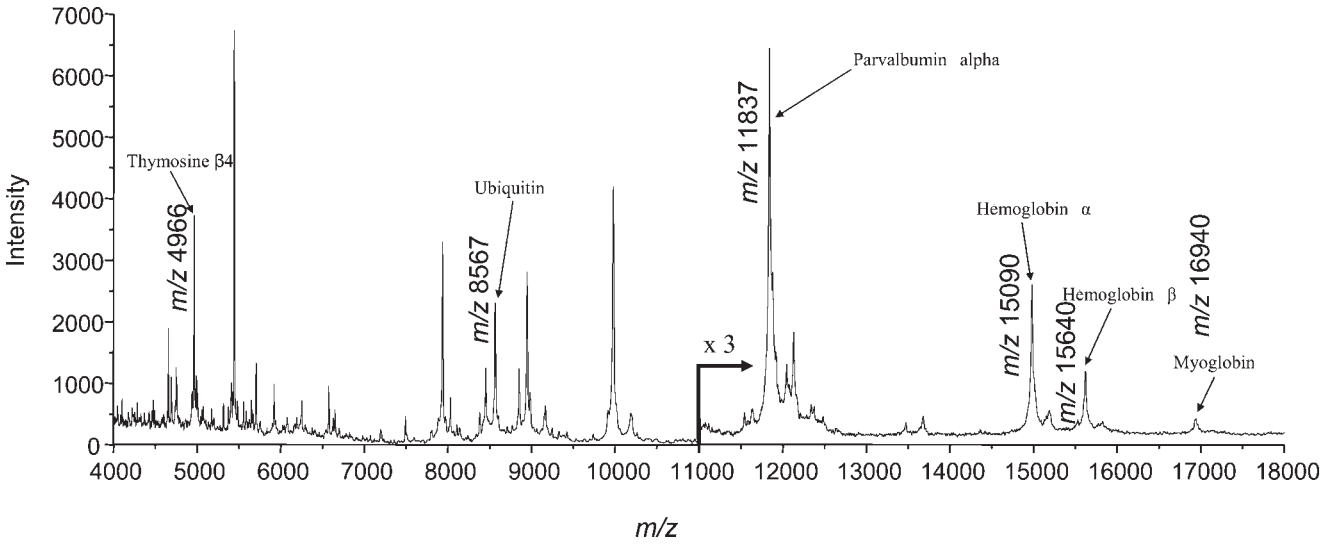

Figure 1. Positive ion MALDI mass spectrum acquired from a mouse leg tissue section, after three successive droplet depositions of the matrix solution (SA, 30 mg · mL^{-1}, in water/acetonitrile, 1:1, v/v), in the linear mode, by accumulating about 1000 shots per matrix spot.

tissue sections. This observation is in good agreement with several other studies, in which two calcium-specific binding sites of parvalbumin α were described.[15–17] Due to its ionic radius being relatively close to that of calcium (Table 1), cadmium was expected to behave in a similar way with respect to this protein. This was clearly the case in the present study, confirming previous investigations performed by NMR spectroscopy.[18,19] As a con-

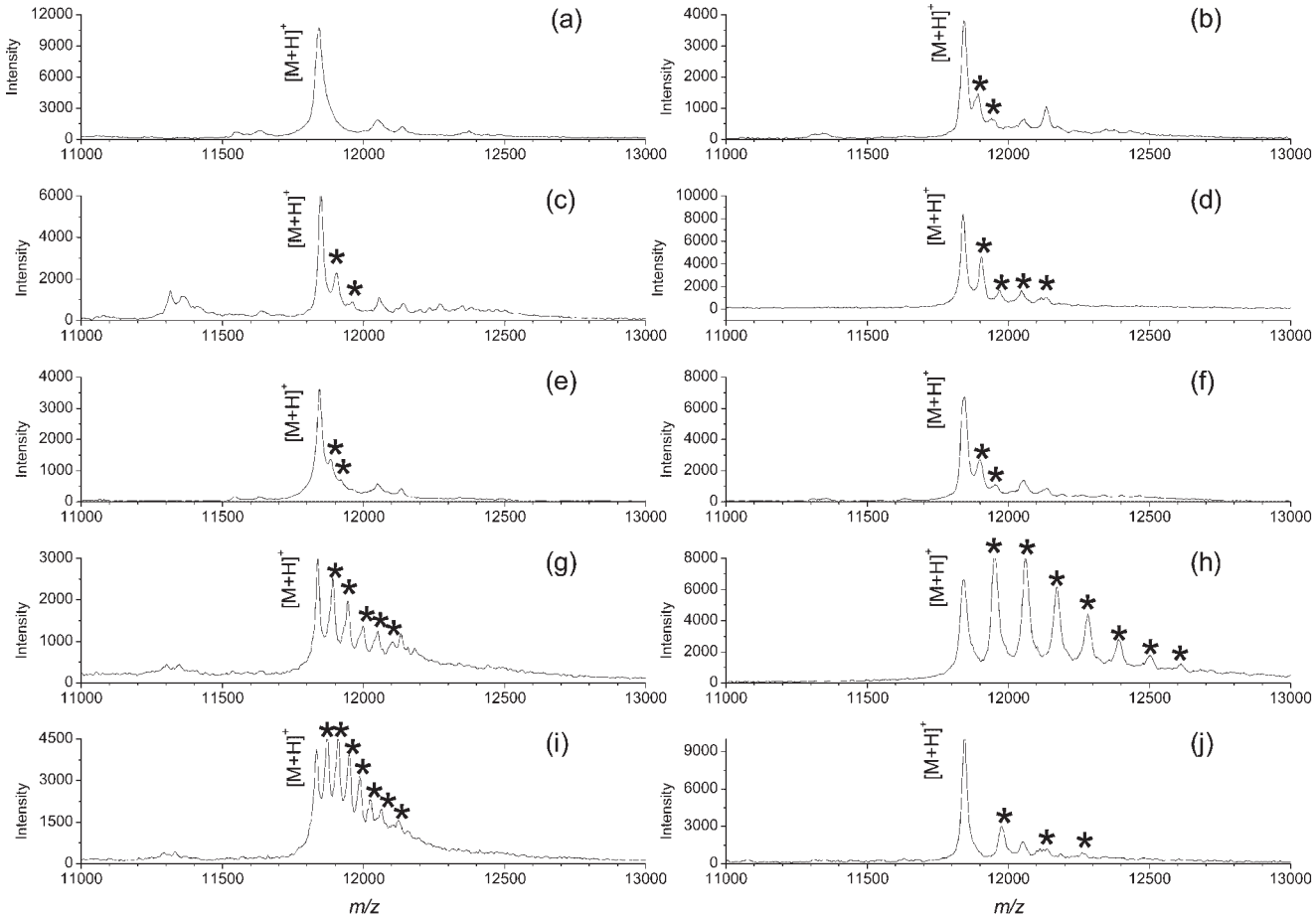

Figure 2. Positive ion MALDI mass spectra acquired from a mouse leg tissue section, after three successive droplet depositions of the matrix solution, in the linear mode, and by accumulating about 1000 shots per matrix spot. The matrix solutions were mixtures (10:1, v/v) of a SA stock solution (30 mg · mL^{-1}, in water/acetonitrile, 1:1, v/v) with solutions containing (a) $AlCl_3$, (b) $CrCl_3$, (c) $NiCl_2$, (d) $ZnCl_2$, (e) $FeCl_2$, (f) $CoCl_3$, (g) $MnCl_3$, (h) $CdCl_3$, (i) $CaCl_2$, and (j) CsCl, respectively, at a concentration of 100 μM in water/acetonitrile (1:1, v/v). Each peak labelled with a star corresponds to a metal adduct.

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Table 1. Ionic radii of the metal cations[22]

Ion	Ionic radius (Å)
Al^{3+}	0.54
Cr^{3+}	0.60
Ni^{2+}	0.69
Zn^{2+}	0.74
Co^{2+}	0.75
Fe^{2+}	0.78
Mn^{2+}	0.83
Cd^{2+}	0.95
Ca^{2+}	0.98
Cs^{2+}	1.67

sequence, it seems that the protein structure could be partially preserved in the tissue section, especially in the absence of any treatment except the matrix deposition, and if the analysis takes place shortly after the drying process.

This is the first time, as far as we know, that it has been possible to detect directly on tissue sections specific interactions between metal ions and a protein molecule. These experiments suggest that it may be possible to confirm the identity of a metalloprotein in such complex samples without any purification step. Because one of the major limitations remains the sensitivity, new techniques, such as the use of automated spotters for the matrix deposition[20] or cryodetectors,[21] are needed to improve the accessible mass range and the detection limit. Further studies are in progress in order to apply this method to the direct analysis of protein/ligand non-covalent complexes on tissue sections.

David Touboul[†], *Alain Brunelle and Olivier Laprévote**
ICSN-CNRS, Laboratoire de Spectrométrie de Masse, Avenue de la Terrasse, 91198, Gif-sur-Yvette Cedex, France

**Correspondence to: O. Laprévote, ICSN-CNRS, Laboratoire de Spectrométrie de Masse, Avenue de la Terrasse, 91198, Gif-sur-Yvette Cedex, France.*
E-mail: laprevote@icsn.cnrs-gif.fr

[†]*Present address: Department of Chemistry and Applied Biosciences, ETH Zürich, CH-8093 Zürich, Switzerland.*

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