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SOME APPLICATIONS OF MASS SPECTROMETRY IN MONITORING PATHOLOGICAL STATES

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In the last decade the applications of mass spectrometry in the medical field have shown a fantastic grown, mainly due on one hand to the development and use of the newest mass spectrometric techniques, on the other to the physician consciousness of the high specificity and analytical power of these new techniques.
In the present communication two experiences of the our group will be described.
The first pertains the use of mass spectrometry in the study of *in vitro* and *in vivo* non enzymatic protein glycation, a process which is of medical relevance either in diabetes or in the end stage renal disease. In particular MALDI/MS and ESI/MS have been shown to be precious tools to investigate this problem, allowing either the accurate definition of the number of glucose molecules condensed or a specific protein or the identification of the preferred glycation sites.
The second topic is related to the use of mass spectrometry to identify possible markers of cancer development. For this aim the plasma and serum protein profiles of a population of healthy subjects, obtained by MALDI/MS, have been compared with those from patients bearing colon-rectal cancer or melanoma. Clear differences were put in evidence, consisting, in the case of patients, of an underexpression of proteins present in healthy subjects and in the production of new polypeptides.
These preliminary results suggest that MALDI/MS can be successfully employed for diagnostic purposes.

1. Introduction

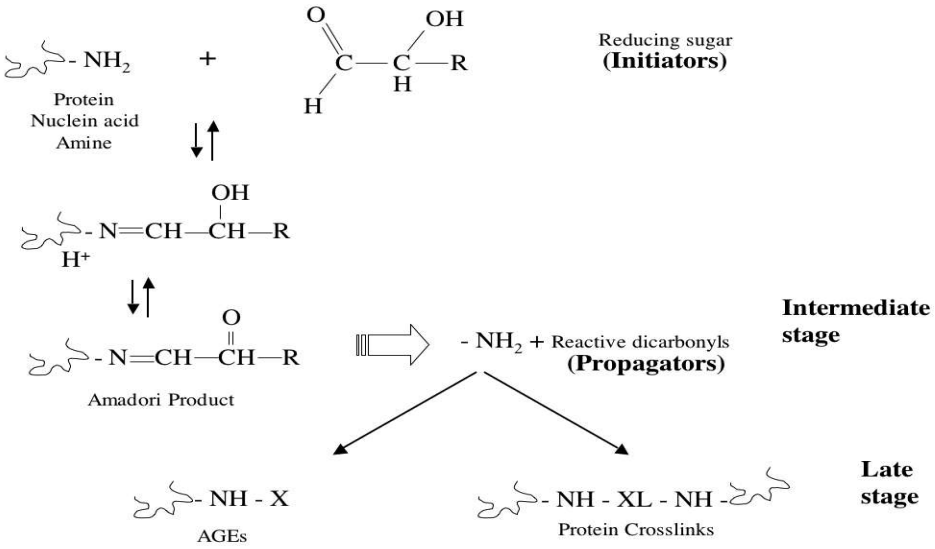
Nowadays mass spectrometry is a fundamental analytical tool in biomedical research, mainly due to the development of instrumental methodologies particularly powerful in the field of proteome. Matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are currently employed for structural characterisation of proteins and peptides, giving valid information on molecular weight and sequence. Here we report some results obtained by our group on two different applications in the fields of diabetes monitoring and of

identification of possible markers of melanoma and colon-rectal cancer development.

2. Glyco-oxidation in diabetes and related diseases

A series of experimental and clinical evidence indicates that hyperglycemia-derived free radicals may represent a common pathway in the pathogenesis of the chronic complications of diabetes, aging and uremia.[1]

The non-enzymatic glycation reaction plays an important role in long term diabetic complication (Fig 1). Briefly, it may be subdivided into three main stages: *early stage,* consisting of the condensation of reducing sugars with the -amino groups of lysine residues of proteins, and the formation of a Schiff base which rearranges into a more stable ketoamine, usually called Amadori product. In the *intermediate stage,* the Amadori product is degraded into a series of carbonyl compounds (glyoxal, methyl glyoxal, deoxyglucosones), which are highly reactive and act as propagators of the reaction. In the *late stage,* these propagators again react with free aminogroups and, through rearrangements, lead to the formation of



advanced glycation end-products (AGE).

Figure 1. The Maillard reaction patway

AGE levels in the body reflect a balance between their formation and catabolism through tissue degradation and renal elimination. Tissue degradation

occurs through endocytosis by macrophages and other cellular systems via receptor-specific or non-receptor pathways. The subsequent proteolysis of AGE determines the formation of low molecular weight AGE-peptides, which are excreted into the urine. Other intracellular protective mechanisms also limit AGE formation, e. g., the glyoxalase system, which converts methyl glyoxal into D-lactate and antioxidant substances.

Bearing in mind the complex and close interactions between glycation and oxidation, AGE accumulation may represent a common pathway, explaining the complications not only of diabetes but also of a series of other diseases as those occurring in uremia and aging (Table 1).

Table 1

Glyco-oxidation: a common pathway for complications of some chronic diseases

Diseases	Mechanisms of damage	Complications
Aging	Oxidative stress Decreased turnover of proteins Insulin resistance	Atherosclerosis Hypertension Renal damage Dyslipidemia Cataract Dementia Osteoporosis
Diabetes mellitus	Hyperglycemia	
Uremia	Oxidative stress Decreased clearance Insulin resistance	

In vitro studies

MALDI was first applied in the study of *in vitro* glycation of bovine serum albumin (BSA), which was incubated in pseudo-physiological conditions (pH 7.35, 37 °C) with glucose at various concentrations (0.2, 2, 5 M) for 0-28 days.[2] A progressive increase in the molecular weight of BSA was observed by comparing the MALDI spectra of unglycated BSA (showing the main peak centered at 66429 Da) with those obtained with protein incubated with glucose. As the condensation of a glucose molecule on a protein determines a mass increase of 162 Da, it is easy to calculate the number of glucose molecules condensing on the protein samples.[55] Analogous results were obtained by *in vitro* incubation of several proteins, such as lisozyme and ribonuclease, with glucose and/or fructose.

In vivo studies

The results of the above studies led us to apply the same technique to *in vivo* study of glycated proteins.[3] MALDI was applied in the evaluation of plasma samples of healthy subjects and patients affected by type 2 diabetes mellitus in varying degrees of metabolic control. It should be emphasized that sample preparation was extremely easy, and consisted simply of passing the plasma through an Amicon membrane (cut-off 10 kDa) to eliminate free glucose and salts,

and then centrifuging it at 3000 rev/min; after discarding the supernatant samples were lyophilized, added to the MALDI matrix, and analyzed directly .

The MALDI spectra of plasma samples from healthy subject show abundant peaks at m/z 66680 Da, due to human serum albumin (HSA), and at 148676 Da, corresponding to IgG. The MALDI spectra from samples of diabetic patients show an increase in molecular weight of the peaks for HSA and IgG. This mass increase (ΔM) is due to glucose molecules condensing on HSA and on IgG. Due to its high abundance, we focused attention on HSA, and the metabolic control parameters (fasting plasma glucose, HbA1c, furosine) and MALDI data for 10 healthy subjects, 10 diabetic patients in good metabolic control and 20 diabetic patients in bad metabolic control are shown in figure 2. In patients with well controlled diabetes the ΔM values range from 439 to 2403 Da, corresponding to the condensation of 3-15 glucose molecules; in patients with well controlled diabetes the values range from 266 to 496, corresponding to 2-3 condensed glucose molecules, and in healthy subjects are practically zero What is to be emphasized is that, whereas plasma glucose, HbA1c and furosine values are quite close in each class of subjects, ΔM values show great differences: particularly in patients with poorly controlled diabetes, great difference among subjects was found, highlithing the differing individual proclivity to glycation.

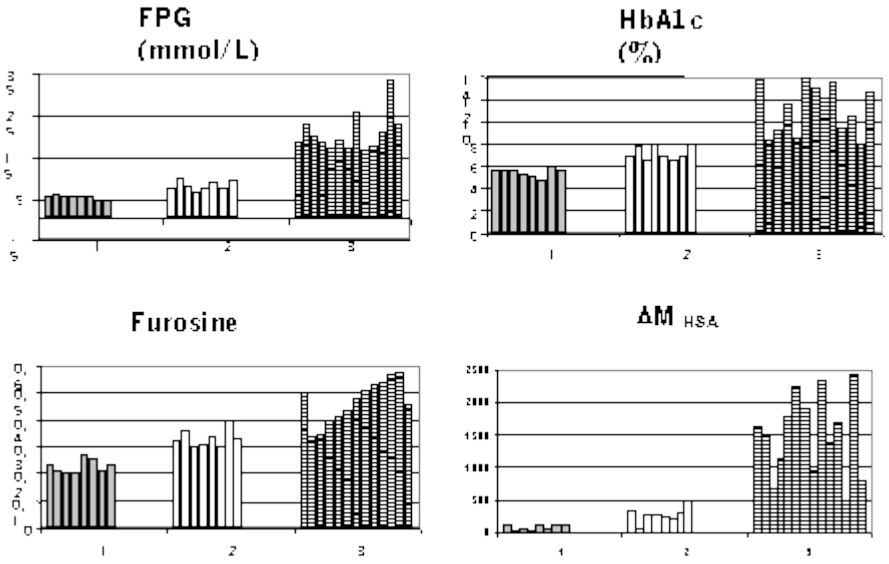


Figure 2. Mean values (±SD) of fasting plasma glucose (FPG), Furosine, HbA1c and ΔM obtained by MALDI measurements on HSA (Human Serum Albumin) in the subject under study. 1 normal glucose tolerant subjects 2 well controlled diabetic patients 3 poorly controlled diabetic patients

Analogous results were obtained when evaluating the number of glucose molecules condensing on IgG.

The glycation level of hemoglobin (HbA1c) is currently used to evaluate the metabolic control of diabetic patients of the 4-6 weeks preceding the test and, results from a series of epidemiologic consider HbA1c as the "gold standard" in the diabetes monitoring. As the procedures used to evaluate HbA1c levels (liquid cation exchange chromatography, colorimetry, isoelectrofocusing, immunoassays), can evaluate the amount of glycated βglobin but cannot determine the number of glucose molecules condensing on the protein, we therefore believe it is of interest to apply the MALDI technique to the study of globin glycation.[4] The MALDI spectrum of the globin fraction from a healthy subject is shown in figure 3. The peaks at m/z 15127 and m/z 15868 are due to protonated non-glycated αand βglobins respectively, while the minor peaks at m/z 15289 and m/z 16030 correspond to protonated glycated α and β globins respectively. When samples from diabetic patients were analysed, the abundance of peaks related to glycated αand βglobins is clearly increased, and the peaks corresponding to the addition of glycation-oxidation products are more evident, as well as peaks corresponding to the addition of 2 glucose molecules.

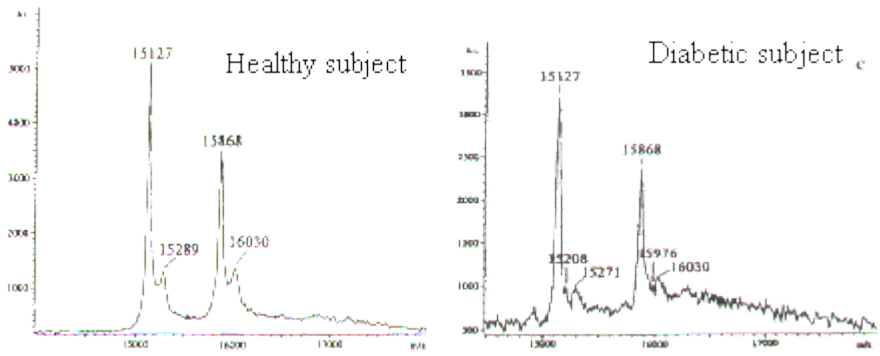


Figure 3. MALDI mass spectra of globin fraction of a healthy subject and a diabetic patient

Studies on AGE peptides [5]

As stated above, degradation of AGE causes the formation of small glycated peptides called AGE peptides, which are filtered and reabsorbed by the proximal tubules and metabolized. Thus, impairment of renal function determines a progressive increase in these peptides in both diabetic and non diabetic subjects. What is to be emphasized is that these compounds are very reactive, so that their

accumulation in plasma and tissues leads to several kinds of damage (binding to lipoproteins, cross-links to collagen) which may explain the continued progression of chronic complications in patients with renal failure. Clinical and experimental data on these peptides have been obtained by isolating these compounds from plasma by ultrafiltration at a molecular mass cut-off of 10,000 kDa.

Considering the importance of structural characterization of these AGE peptides, we undertook an investigation based on *in vitro* glycation of BSA, treatment of samples with proteinase K, and subsequent analysis of the digestion products by means of various mass spectrometric approaches such as MALDI/MS, LC/ESI/MS and LC/ESI/MS/MS. Results of these studies showed that there are clearcut differences between the digested mixtures of glycated and unglycated proteins, although no structural information could be obtained on the species present in the glycated proteins.

Taking into account the fact that proteinase K cleaves peptide bonds adjacent to the carbonylic group of aliphatic and aromatic aminoacids, we decided to use a more specific enzyme, i.e. trypsin, to identify possible AGE peptides. Tryptic digestion products of *in vitro* glycated HSA were analyzed by MALDI/MS, LC/ESI/MS, LC/ESI (MS/MS and Fourier Transform Mass Spectrometry (FTMS) for accurate mass measurement. We were able to identify about 20 glycated peptides in the hydrolized mixture of glycated proteins, and their structure was postulated on the basis of accurate mass measurements.

On the basis of these results we undertook an *in vivo* study, and the low molecular weight peptide fraction of plasma samples from 10 healthy subjects, 10 poorly controlled diabetic patients and 10 end-stage kidney disease patients were analized by MALDI and LC/ESI/MS.

Results showed that none of the glycated peptides identified in the *in vitro* experiments were found in serum samples: this may be explained both by the different digestion occurring *in vivo*, and by the high number of serum proteins. However, clearcut differences were found among the serum samples of healthy, diabetic and nephropathic subjects. The data highlight the need of a more effective prefractionating of the low molecular weight serum peptides before mass spectrometric analysis, and work is in progress along these lines.

3. Search of cancer markers in plasma

The application of new technologies in the cancer research would lead, in principle, to important effects on public health and to a detailed study on the mechanism(s) responsible for cancer development. In this frame the structural identification of specific markers of the disease is essential, and the new mass spectrometric methodologies developed for the study of proteoma seem to be highly promising. By this approach interesting results have already appeared in

literature.

In an early investigation the plasma protein profiles of samples coming from melanoma patients were studied by selecting the plasma low mass proteins and peptides and by their analysis by MALDI/MS.[6] Clear differences were observed with respect to plasma proteins of healthy subjects: in particular, in the case of melanoma patients, the appearance of new peptides and the disappearance of species present in healthy subjects were observed (Fig 4). The most frequently detected species in patients were those at m/z 14040 (14 cases of 27, i.e. 52%) and 13874 (10 cases of 27, i.e. 37%). Among control subjects, only the species at m/z 14061 (5 cases of 10, i.e. 50%) has been detected in this range. It should also be noted that the species at m/z 28308 and 29133 for healthy subjects, and 22999 for melanoma patients, were observed in only one subjects each, and therefore are of negligible relevance. These data suggest that plasma proteins with molecular weight in the range 10000-15000 Da are of particular interest in order to characterise patients with malignant cutaneous melanoma.

Looking at valid results so obtained, we thought of interest to apply the MALDI/MS-based approach to the study of plasma samples from patients bearing colon or rectum cancers and to compare the peptide pattern so obtained with that from healthy subjects, with the final aim to put in evidence some peptides useful as markers of the disease.[7]

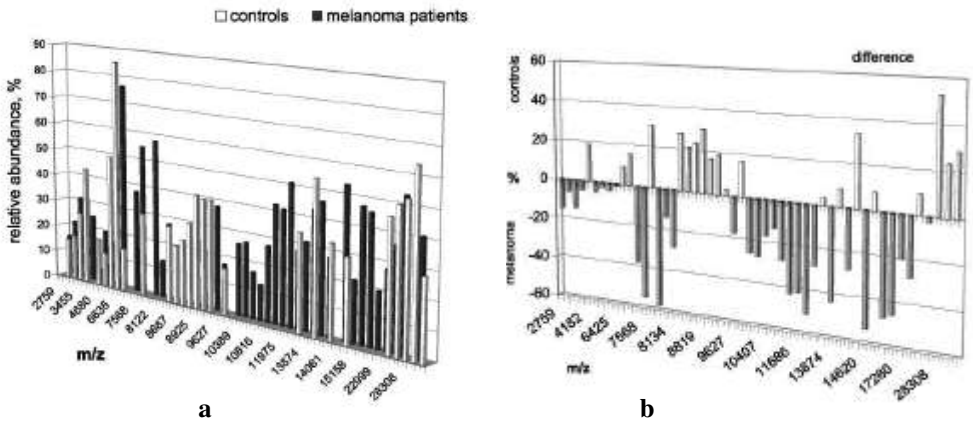


Figure 4. (a) Mean values of relative abundances of serum protein ions in melanoma patients and healthy controls at various m/z values. (b) Difference between the relative abundances of peaks in the two groups.

For this preliminary study plasma samples from 9 healthy subjects, 10 patients affected by colon cancer (stage IV) and 11 patients bearing rectum cancer,

randomly chosen, were analysed. The cancer patients were not subjected to chemotherapy or radiotherapy before the blood withdrawing. The patients gave their formal informed consent for the study, accordingly to the Helsinki declaration.

The plasma samples were diluted with saline solution (volume ratio 1:3) and centrifuged for 40 min at 4000 g (8°C) in Centricon tubes at 30000 Da cut-off. Before MALDI/MS analysis, the filtrates were desalted and purified by ZipTip $_{C18}$ pipette tips (Millipore), following the procedure described in the ZipTip user guide.

First of all the reproducibility of the data so obtained was tested by analysis of the same plasma sample from a healthy subject, divided in eight aliquots. Each aliquot was separately treated and analyzed as above described. In all MALDI spectra the most of the same peaks were present, with only minor changes in relative abundances. The coefficient of variation (%) of relative abundances was in the range 9-27 %. Furthermore, it was thought of interest to calculate for the different couples of spectra the discrepancy factor D_{ij}, defined by Crawford and Morrison as the sum of the absolute value of the differences in relative abundances (RA) of the same ions in spectra of two different samples. Their values are in the range 31-46 (mean ± standard deviation: 39±5). It is to emphasize that identical spectra would lead to a D_{ij} value equal to zero, and that small differences in spectra reflects heavily in D_{ij} values. Thus, as an example, by well reproducible methods, as for example EI, D_{ij} values in the range 5-30 are usually obtained for different spectra of the same sample. Interestingly, just at first sight, clear differences were observed among the MALDI spectra of plasma samples from healthy subjects, colon cancer and rectum cancer patients, as shown by spectra, typical of the three classes of subjects, reported in Figure 5.

However, in order to evaluate the real significance of these differences, some data elaborations were performed. First of all the intra-class D_{ij} values (i.e. those related to MALDI spectra from plasma sample from the same class of subjects) were calculated. Thus, the D_{ij} value related to MALDI spectra of plasma samples from healthy subjects were found in the range 144-768 (mean±SD: 363±229), while for the sets of samples from rectal and colon cancer patients D_{ij} values in the ranges 264-515 (mean±SD: 399±98) and 192-951 (mean±SD: 576±287) respectively were determined. These values give account for the individual variability of low molecular weight proteins molar ratio in the three sets of plasma samples.

All the ionic species found in the three groups of people (healthy subjects, colon cancer patients and rectum cancer patients) were considered for the clustering procedure (Figure 6). In the Figure 6 all the mass data of all the samples have been overlapped and the clustering analysis approach, optimized by using four clustering criteria, produced a grouping within all the data that seemed to indicate

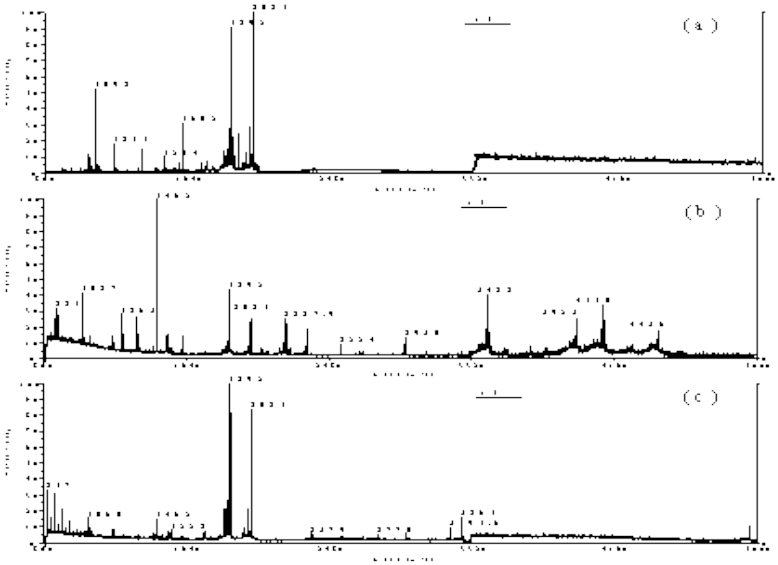


Figure 5. Typical MALDI mass spectra of plasma from patients affected by colon cancer (b) or rectum cancer (c), in comparison with the spectrum of an healthy subject (control, a)

four areas in the plot of m/z versus relative abundance among which two can be validly employed for diagnostic purposes. The clusters indicated by the ovals in Figure 6 include a large number of patients' data, therefore appearing to be

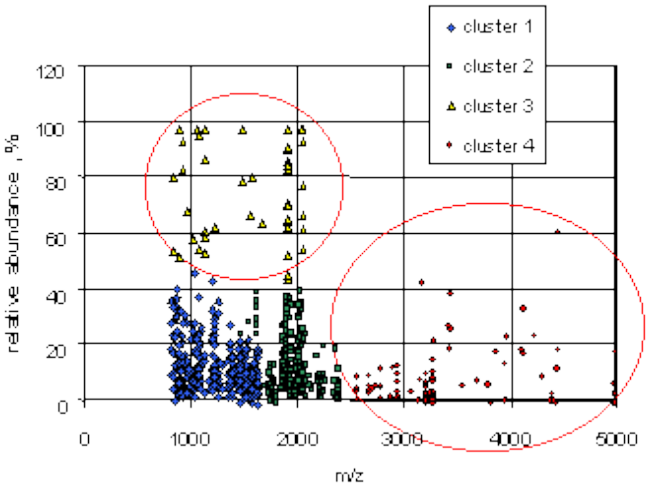


Figure 6. All the ionic species found in the three groups of people (healthy subjects, colon cancer patients and rectum cancer patients) and the result of cluster analysis of the data according to 4 clustering criteria (Ward's method); symbols of the same type indicate belonging to one of the determined clusters. Areas outlined by the ovals indicate the clusters including many of the patients' data, therefore of possible interest for characterizing the disease

informative about the disease. It should be noted however that the data dispersion is high; overlapping of ionic species belonging to the three groups was found, and ionic species not in common among different spectra were clearly shown.

These data allow to evidence a high number of peptides not detected in healthy subjects which are present in colon and rectum cancer patients. Some of them are common between the two groups of cancer, while some other appear to be specific for the colon cancer only.

In conclusion, our data suggest that some ionic species detected by MALDI/MS in the plasma of patients affected by colorectal cancer, could be of interest for a more complete molecular classification of the disease, and might provide new information in the development of clinical diagnostic and prognostic tools. Of course, these represents only preliminary data on low molecular weight plasma peptides. They are, in our opinion, promising but further studies on a larger set of plasma samples and using different fractionation procedures are required.

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