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Review

Some views on proteomics in diabetes

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Abstract

Mass spectrometry has been widely used in the field of diabetes. The development of new ionization methods and the effective coupling of mass spectrometry with liquid chromatography have enabled the protein modifications due to glycation processes to be investigated. Matrix assisted laser desorption/ionization mass spectrometry (MALDI/MS) has been used to evaluate the degree of glycation of specific plasma proteins. In contrast, the classic proteomic approach has been used to identify glycation sites and condensed sugar modifications. The same methods have been applied to studies on urinary protein profiles, enabling changes due to the development of long-term, diabetes-induced nephropathy to be identified. Published studies demonstrate that mass spectrometry is an important analytical tool for monitoring diabetes, capable of providing physicians with a new, more complete view of the physiopathological changes occurring as the disease develops.

Keywords: advanced glycation end-products; diabetes complications; electrospray ionization mass spectrometry (ESI/MS); matrix assisted laser desorption/ionization (MALDI); protein glycation; urinary protein profile.

Introduction: the proteomic era

The human genome has been completed (1) and it has become clear that the genome provides the basis for evaluating what is possible, but it cannot provide crucial information about what there ''*is*'' in a living organism. For this reason, genomics can be considered to be responsible for the birth of proteomics (2), which can be defined as the study of the whole set of proteins expressed by the genome. Proteins are in turn responsible for the production of a set of small molecules responsible for the operation of living organisms, usually called the metabolome (3).

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The genome, proteome and metabolome thus represent three different, but intimately connected aspects of a living system. They enable the identification of its molecular pathways. The great complexity of these systems made them difficult to thoroughly elucidate, and it is only recently, with the development of highly-specific and sensitive analytical methods (4), that an in-depth knowledge has been possible, and some interesting results have been obtained concerning their interconnections.

Aside from these general aspects, the availability of these new analytical approaches has enabled changes in the physiological protein profiles induced by the presence of specific diseases to be investigated, particularly with respect to protein-protein interactions and post-translational modifications that cannot be addressed by genomics or transcriptomics (5).

Today, physicians consequently have new diagnostic tools at their disposal, but their validity needs to be accurately and critically evaluated.

It has been well demonstrated that a pathological state is reflected by a different plasma and urine protein profile, with the presence of new proteins and the underexpression of others found in absence of disease.

Leaving aside the possible changes at the genome level, such as those occurring in cancer development, the changes in protein profiles in pathological conditions can be mainly attributed to two different reasons:

i. activation of the synthesis of new proteins due to an immune and/or anti-inflammatory response to the disease. However, it is important to bear in mind that pro-inflammatory reaction states can prevail in many diseases;
ii. the synthesis of proteins expressed in healthy conditions may be inhibited and new proteins produced in pathological conditions may be the target of enzymatic digestion.

The result of these two aspects is that the protein profile observed in illness shows the presence of new proteins/peptides and the disappearance of some of those observed in health.

Proteomics: power and limits

The analytical approach that gives the most specific information in the field of proteomics is certainly the one based on mass spectrometry (MS) (6). This technique can provide data on protein mass and abundance and, in some cases, on the amino acid sequence (thus enabling the protein's identification), but it provides no direct information on enzyme activity. MS represents the final step in a series of sample treatments designed to enable the analysis, from a qualitative

or quantitative standpoint, of specific proteins in biological samples. Laboratory steps are needed prior to MS analysis, and their validity and reproducibility must be accurately tested (4). Once obtained, the samples must be cooled rapidly to inhibit or at least strongly reduce the protease activity. Some protease inhibitors can be used for this purpose but this generally increases the complexity of the biological sample. The storage of a biological sample is also important to ensure that analysts investigate the proteins present in vivo and not their enzymatic digestion products. Samples stored at low temperatures must only be thawed once because sequential heating-cooling steps lead to severe protein degradation. A biological sample can be divided between different vials to prevent this.

Many different approaches have been proposed for the preparation of samples for MS analyses (4): Figure 1 shows the two most widely used. In the first (on the left in Figure 1), the protein mixture is first treated by electrophoresis (mono- or bi-dimensional) to separate the different proteins contained in the mixture. It would be better to define this step as a ''partial'' separation procedure because neither mono- nor bi-dimensional electrophoresis exhibits sufficient resolution to completely separate the different proteins. What is generally seen is more than one protein in a band (in the case of mono-dimensional electrophoresis) or spot (in 2D conditions). The denaturing conditions of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) must also be considered.

The best results can be obtained by capillary zone electrophoresis (CZE) (7), but this approach is more expensive than 1D and 2D electrophoresis, both in terms of the cost of the instrumentation and because of the lengthy analysis times.

Obtaining intact protein from a 1D or 2D electrophoresis gel is a difficult task, due mainly to the small quantity of the analytes. The proteins contained in a band or spot are generally digested enzymatically and the structures of the peptides produced by the digestion are then identified. This identification step can be conducted using product ion mass spectrometry (MS/MS) (8) of protonated molecules of the analytes generated by electrospray ionization (ESI) (9). The

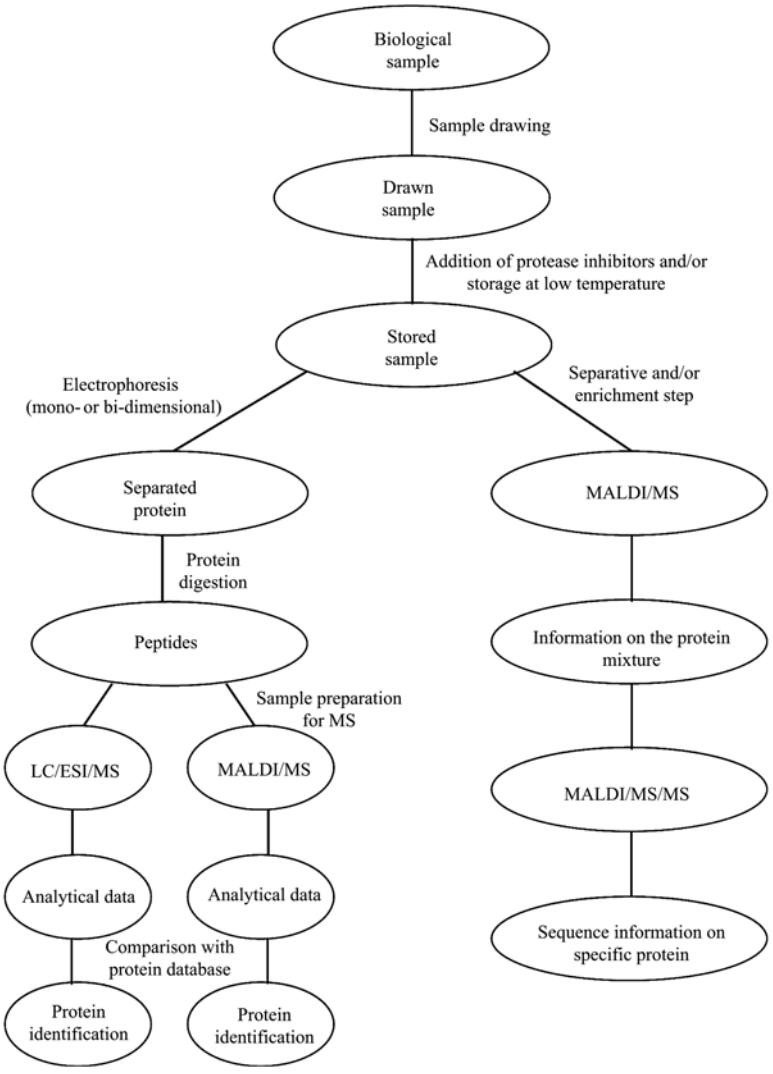

Figure 1 Proposed approaches for proteomic investigations using mass spectrometry.

quality of the results depends on the mass spectrometric resolution (10). In high-resolution conditions, structural assignment is based not only on the collisionally-induced decomposition products (MS/MS), but also on their elemental compositions that are deduced from accurate mass measurements. This approach thus provides information on some of the protein substructures (peptides) but not directly on the original protein, and this latter information must be obtained by comparing the tryptic fragments with protein databases to obtain a list of possible proteins with experimentally determined amino acid sequences (4).

In recent years new MS analytical approaches have become available and have proved highly effective in proteome studies. Their validity was recognized by the scientific community with the awarding of the Nobel Prize in 2002 (11).

The first method, called electrospray ionization (ESI) (9), is based on the protein solution interacting with strong electrical fields, leading to ion production through protonation reactions. Ionic species containing a different number of protons are generated, depending on the protein's dimensions, and the resulting ionic cluster is deconvoluted using suitable computer programs, enabling the molecular weight of the intact protein or peptide to be determined.

A separation step [e.g., liquid chromatography (12) or capillary zone electrophoresis (CZE) (13)] is required for the analysis of the complex mixtures of proteins/peptides. However, the ESI spectrum otherwise becomes too complex due to the ion clusters originating from the different molecular species overlapping.

An alternative to ESI, which has been widely used in protein investigations, and usually called matrix assisted laser desorption/ionization (MALDI) (14), is based on the interaction of a laser beam with a solid-state sample consisting of a suitable matrix (usually variously substituted aromatic acids) and traces of analyte (the typical analyte: matrix molar ratio is in the order of 1:10,000). This interaction leads to the rapid vaporization of a microvolume of a solid sample and the ionization of matrix molecules. Ion-molecule reactions take place in the high-density plume generated, with high-yield production of protonated $[M+H]^+$ species of the analyte.

Thanks to the production of a singly-charged species for each analyte, MALDI can be used to analyze protein/peptide mixtures directly. However, it should be emphasized that the intensity of the ratio of the ions detected does not necessarily correspond to the molar ratio of the different components, due to the occurrence of ion suppression effects. In other words, MALDI can provide a direct qualitative view of a protein mixture, but an accurate set-up of the method is required for quantitative evaluations. The results obtained by both ESI and MALDI in the field of diabetes are described in detail in the examples given below.

Another technique used in the proteome field is surface enhanced laser desorption/ionization (SELDI) (15). In SELDI, the protein mixture is spotted onto a surface modified with a specific chemical functionality. Some proteins in the sample bind to the surface, while others are removed by washing. After washing the spotted samples, the matrix is applied to the surface and allowed to crystallize with the sample peptides. The binding to the SELDI surface acts as a separation step, and the subset of proteins that bind to the surface are easier to analyze. Common surfaces used in this context include: weakly positive ion exchange, hydrophobic surfaces (similar to those used in C_6–C_{12} reverse phase chromatography), metal-binding surfaces, and strong anion exchangers. Surfaces can also be functionalized with antibodies, other proteins, or DNA.

Proteomics and diabetes: two different approaches

Reducing sugars react with amino groups following a pathway studied more than a century ago by Louis Camille Maillard (16). Briefly, this reaction can be divided into three main stages. An *early stage* consists of the condensation of reducing sugars with the ε-amino groups of lysine residues of proteins, and the formation of a Schiff base that becomes rearranged into a more stable ketoamine, usually called the Amadori product. In the *intermediate stage*, the Amadori product is degraded into a series of carbonyl compounds (glyoxal, methylglyoxal, deoxyglucosones), which are highly reactive and act as propagators of the reaction. In the *late stage*, these propagators again react with free amino groups and, through rearrangements, lead to the formation of advanced glycation end products (AGEs) (17).

It is worth noting that Maillard suggested the important role of this reaction at the biological level, given the presence of amino groups (i.e., reacting sites) in proteins and other biological substrates. As a matter of fact, in vivo non-enzymatic protein glycation has proved to be active at the systemic level with the production of reactive species released by glycated proteins, and the formation of cross-linked proteins at the tissue level (18). It has been suggested that this latter mechanism justifies the morphological changes in tissues observed with aging and diabetes. This is due to the long time scale of the reaction in the former case, and to its stoichiometry in the latter (high circulating and cellular glucose concentrations). In this context, it is worth emphasizing that glycation of skin collagen has proved capable of predicting the risk of future progression of diabetic complications (nephropathy and retinopathy) in patients followed in the Diabetes Control and Complication Trial (19).

The determination of protein glycation levels can be a useful diagnostic tool, and the glycation level of hemoglobin (HbA_{1c}) is currently used as a valid parameter of metabolic control (20). In fact, the glycation level of a circulating protein with a known half life enables us to evaluate an individual's exposure to the mean glycation level. It is worth adding that two studies have confirmed HbA_{1c} as the ''gold standard'' for assessing metabolic control: these two randomized trials, the Diabetes Control and Complication Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), demonstrated a close correlation between glycemic control (as assessed by measuring HbA_{1c}) and the

risk of the onset and progression of the complications of diabetes (21, 22).

Globin was chosen as the target compound because extensive investigations have been conducted on the relationship between its glycation level and the long-term complications of diabetes.

Other markers currently used at the clinical level for glycemic control are the reactive species released during the Maillard reaction (e.g., glyoxal, methylglyoxal, deoxyglucosone) (18). The glycation level of specific proteins (in terms of the number of condensed glucose molecules and the reactive sites engaged) can also be considered as a new and effective clinical tool.

Glycation level of intact proteins

The glycation level of specific proteins can easily be determined by MALDI: the mass difference between glycated and non-glycated protein (ΔM), divided by 162 (the molecular weight of a condensed glucose unit) gives us the minimum number, n, of the glucose molecules condensed on the protein.

$$n = \frac{\Delta M}{162}$$

This is the minimum number because dehydration-oxidation processes may take place on the condensed sugar, consequently lowering its molecular weight.

In vitro and in vivo glycation To verify the power of the MALDI method in protein glycation studies, some preliminary investigations were conducted on glycated proteins in vitro. BSA, lysozyme and RNase were incubated in pseudo-physiological conditions with glucose at different concentrations (23–25). Samples were obtained after different reaction times. The results unequivocally showed that glycation processes had occurred. The general trend observed on plotting the number of condensed glucose units vs. the reaction time indicates that a ''plateau'' was reached; the height of which depends on the glucose concentrations.

This is consistent with the Maillard reaction pattern, which describes the release of the glucose molecule in the intermediate phase (as well as its dehydrated and oxidized species), which in turn can further react with the protein. In other words, the glycation reaction is expressed by a chemical equilibrium, with a constant that depends on the glucose concentration: the higher the glucose concentration, the higher the final protein glycation level.

Once the power of MALDI in describing glycation levels of glycated proteins had been demonstrated in vitro, the same approach was applied to samples in vivo, focusing on plasma proteins. It is important to note that more than 15,000 different proteins and peptides occur in plasma, in very different concentrations, ranging within five orders of magnitude. This fact, combined with the presence of salts and low-molecular-weight compounds, may make the direct analysis of plasma samples a difficult task. In fact, just passing the plasma sample through an Amicon membrane to remove the free glucose and salts, and then centrifuging at 3000 rpm was sufficient to obtain an analytical sample suitable for MALDI analysis (26). An example of the plasma fingerprint obtained for a healthy subject is shown in Figure 2a. As expected, the most abundant peak, at m/z 66680, is due to protonated human serum albumin (HSA), while the ions at m/z 148676 and 79679 are attributable to IgG and prothrombin, respectively. Two questions arose based on this analysis: are the glycation processes occurring in samples in vivo the same as those observed in vitro? And does the above-described glycation-deglycation equilibrium exist at systemic level?

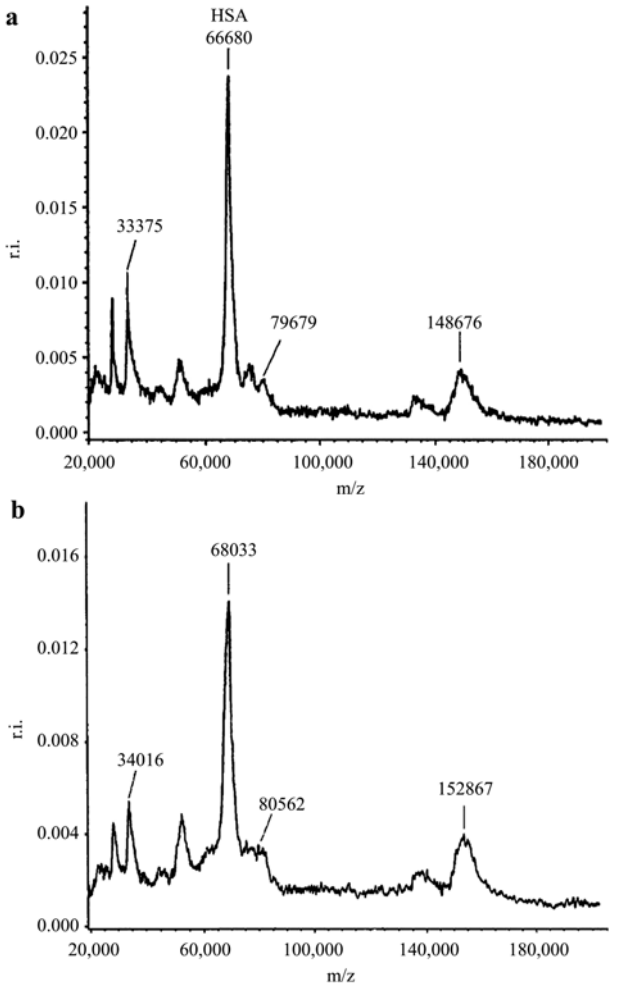

Figure 2 MALDI spectra of a plasma protein fraction obtained from: (a) a healthy subject.
(b) A poorly controlled diabetic patient. (Reprinted from Lapolla A et al. A new effective method for the evaluation of glycated intact plasma proteins in diabetic subjects. Diabetologia 1995;38: 1076–81. Reproduced with the permission of Springer-Verlag.)

Given its abundance, our attention focused first on HSA. Figure 2 compares the MALDI spectra of plasma samples from a healthy subject, (a) and a poorly controlled diabetic patient, (b) the HSA peak in the latter case shifts to m/z 68033, indicating an increase in molecular weight of 1393 Da by comparison with the healthy subject, due to the condensation of at least eight glucose molecules on HSA.

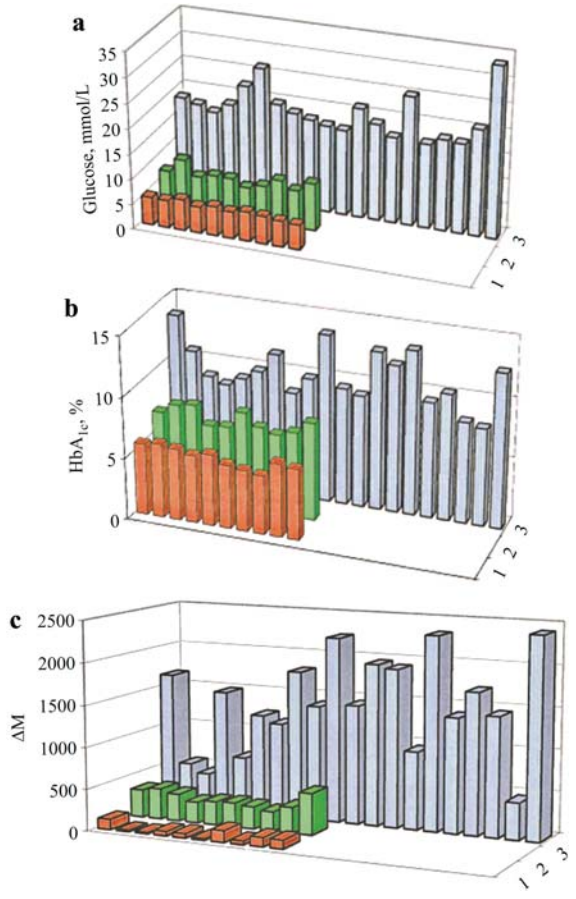

Figure 3 ΔM values obtained by MALDI measurements on HSA from (red; front row) 10 healthy subjects; (green; middle row) 10 well-controlled diabetic patients; (blue; back row) 20 poorly controlled diabetic patients (c) compared with fasting plasma glucose and HbA_{1c} (a and b, respectively).
(Reprinted from Lapolla A et al. A new effective method for the evaluation of glycated intact plasma proteins in diabetic subjects. Diabetologia 1995;38:1076–81. Reproduced with the permission of Springer-Verlag.)

Next, fasting plasma glucose (FPG), glycated hemoglobin (HbA_{1c}), and serum furosine were evaluated in type 2 diabetic patients and normal subjects, also taking plasma samples for MALDI analysis (26). The results obtained from the MALDI analyses for FPG, furosine (representing an indirect measure of the albumin glycation level), and ΔM are summarized in the histograms in Figure 3. The MALDI data show that while a mass increase corresponding to the condensation of 2–3 glucose units is observed in well-controlled diabetic patients, this number is in the range of 3–15 in the case of poorly controlled patients.

It is worth noting that the values observed in the same classes of subjects were quite homogeneous for FPG and furosine, while the ΔM value in poorly-controlled diabetic patients was highly variable. This could be explained by two different mechanisms: the low glycation level observed in some patients could be due either to a more effective glycated protein digestion, or to the activity of a de-glycating enzyme in these individuals (26–28). In the latter case, fructosamine 3-kinase – a 34-kDa protein expressed in all human tissues, but particularly in diabetes-susceptible organs (such as kidney, heart and nervous tissue) (29) – is involved in controlling intracellular non-enzymatic glycation. The enzyme reverses this process by phosphorylating fructoselysine residues to fructoselysine-3-phosphate (FL3P) at the expense of ATP (30). This process destabilizes the fructoseamine linkage, leading to spontaneous FL3P decomposition into lysine, 3-deoxyglucosone, and inorganic phosphate (31). Fructosamines and AGEs can impair the function of enzymes and structural proteins (29), and are thought to participate in the pathogenesis of long-term complications of diabetes (32); FN3K can thus be considered a repair enzyme.

Plasma protein glycation: globulins and globins

MALDI/MS was also applied to IgG, identifying a mass increase up to 4000 Da in poorly controlled diabetic patients, corresponding to the condensation of at least 25 glucose molecules on the protein (33).

In principle, glycated proteins would exhibit different functionality compared with that of unglycated proteins. However, this point is still widely debated due to the scarce and scattered data available on the number and position of glycated protein sites, and their relationship with biochemical and clinical data. Within this framework, investigations have been devoted to verify whether immunoglobulin glycation contributes to a greater susceptibility to infections. Some studies show that incubating IgG at high glucose concentrations leads to a reduced complement-fixing (Fc) activity (34). A similar approach, using IgM, showed that glycation takes place mainly on its fragment antigen-binding (Fab)-like fragments (35). Other studies have shown disparate results (36–38). MALDI data on the intact protein fraction enable the total number of glucose molecules condensed on IgG to be determined, but they were unable to identify the glycation sites responsible for any different Fab and/or Fc component activity (34).

This aspect was investigated by incubating standard IgG in pseudophysiological conditions with 0.5 M glucose for 28 days. A mass increase of 4910 Da, compared with the unglycated protein, was observed, corresponding to the addition of 30 glucose molecules on the native protein. Enzymatic digestion of native and glycated IgG with papain showed that 20 glucose molecules condense on the Fab portion of IgG, and 10 on the Fc portion. Similar results were obtained by incubating the plasma protein fraction of a healthy subject with 1 M glucose in pseudophysiological conditions for 28 days (39).

Computer assisted molecular modeling of IgG was used to obtain further evidence of the above results. It was assumed that the lysine residues most reactive towards sugar were those whose structures were more exposed due to their higher probability of interacting with glucose. The solvent-accessible surfaces (SAS, $Å^2$) for each lysine were therefore calculated, and their distribution on the IgG molecule shows that Fab fragments are more prone to react with glucose, particularly in their light- and heavy-chain variable regions

(LC and HC). LC and HC are important structural moieties in the molecular distinction between antibody and antigen (39).

Considering the high specificity of MALDI analysis for glycated proteins, it seemed worthwhile to investigate hemoglobin glycation. In the 1980s it was shown that glucose can react with α- and ε-amino groups in the major hemoglobin components (40). As discussed above, study of the glycation of a protein with a known half-life gives reliable diagnostic data for evaluating an individual's exposure to hexose. We focused on the formation of A_{1c} hemoglobin (HbA_{1c}) originating from the addition of glucose to the valine α-amino group at the terminus of the hemoglobin β chain.

The current assays for measuring A_{1c} are based on two principles, i.e., the charge and the structural differences between hemoglobin components, and these are summarized in Table 1. HbA_{1c} acquires an extra negative charge when glucose becomes attached to the N-terminal valine of the β chain. The lower isoelectric point is exploited by ion exchange chromatography because the HbA_{1c} accelerates faster in a cation-exchange resin. The concentration of hemoglobin is measured using a spectrophotometer and quantified by calculating the area under each peak of the chromatogram by comparison with a calibrated chromatogram (41). Cation exchange by HPLC is currently the most widely used assay method for measurement.

In 1992, a survey by the College of American Pathologists reported considerable discrepancies in the results of different A_{1c} assays. For the same blood sample, A_{1c} results ranged from 4% to 8.1% (42). Along with the results of the DCCT (21), this survey highlighted the need to standardize A_{1c} measurements in order to improve diabetes management.

The Working Group on HbA_{1c} standardization of the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) developed a reference method (43). In the first step, hemoglobin is cleaved into peptides by the enzyme endoproteinase Glu-C. Next, the glycated and unglycated N-terminal hexapeptides of the resulting β-chain are separated in a second step and quantified by HPLC and ESI mass spectrometry or, alternatively, using a 2D approach, by HPLC and capillary electrophoresis with UV-detection.

The two methods produce identical results. HbA_{1c} is measured as the ratio between the glycated and unglycated hexapeptides. Calibrators consisting of mixtures of highly-purified HbA_{1c} and HbA_0 are used. The analytical performance of the reference method was evaluated by an international network of reference laboratories, which reported excellent results (43).

As described above, the methods currently used for HbA_{1c} measurements determine the amounts of glycated vs. unglycated β-globin, although the chromatographic conditions do not allow for the separation of α- and β-globins (44, 45). To verify the power of MALDI for this evaluation, globins were prepared according to the usual procedure, and a study was conducted to compare MALDI with the liquid chromatography measurements described above (39). A typical MALDI spectrum of the globin fraction of a diabetic patient with no chronic complications is shown in Figure 4b. The two most abundant peaks, at m/z 15126 and 15866, correspond to protonated α- and β-globins, respectively. However, there are many other peaks in the spectrum which can be attributed to modified globins, together with various adducts. Glycated globins give rise to the ions at m/z 16028 and 15288 (β and α, respectively). These two species were also found in the spectra of globin fractions from healthy subjects (Figure 4a), but in less abundance. Some peaks, detected mainly in diabetics, are related to glyco-oxidation processes, such as those at m/z 15225 and 15964 (Figure 4b, c).

Type 2 diabetic patients with different degrees of metabolic control were the object of an investigation conducted using MALDI (46). The percentages of glycated and glyco-oxidated products of α- and β-globins were calculated with respect to the unglycated α- and β-globins. The results were plotted against the HbA_{1c} values obtained using the liquid chromatography method. The percentage of simply glycated β-globin obtained by MALDI vs. HbA_{1c} values is plotted in Figure 5a. It is apparent that the straight line does not cross the origin, but intercepts the x-axis at an HbA_{1c} of approximately 5%, indicating that MALDI data relating to simply glycated β-globin levels are generally lower than those obtained by LC/HbA_{1c} measurements. A similar result was obtained when plotting the sum of the percentages of simply glycated α- and β-globins against HbA_{1c} values (see Figure 5b). Again, the straight line crosses the x-axis at an HbA_{1c} value of approximately 5%. The best results were achieved

Table 1 Current assays for measuring HbA_{1c}.

Assay	Principle	Disadvantage	Advantage
Ion-exchange chromatography (HPLC)	Glycated Hb has a lower isoelectric point and migrates faster than other Hb components	Variable interference hemoglobinopathies, HbF, and carbamylated Hb	Can inspect chromatograms for Hb variants
Boronate affinity	Glucose binds to m-aminophenylboronic acid	Measures not only glycation of N-terminal valine on β chain, but also β chains glycated at other sites and glycated α chains	Minimal interference from hemoglobinopathies, HbF, and carbamylated Hb
Immunoassays	Antibody binds to glucose and between four and 10 N-terminal amino acids on β chain	Affected by hemoglobinopathies with altered amino acids on binding sites, some interference from HbF	Not affected by HbE, HbD, or carbamylated Hb

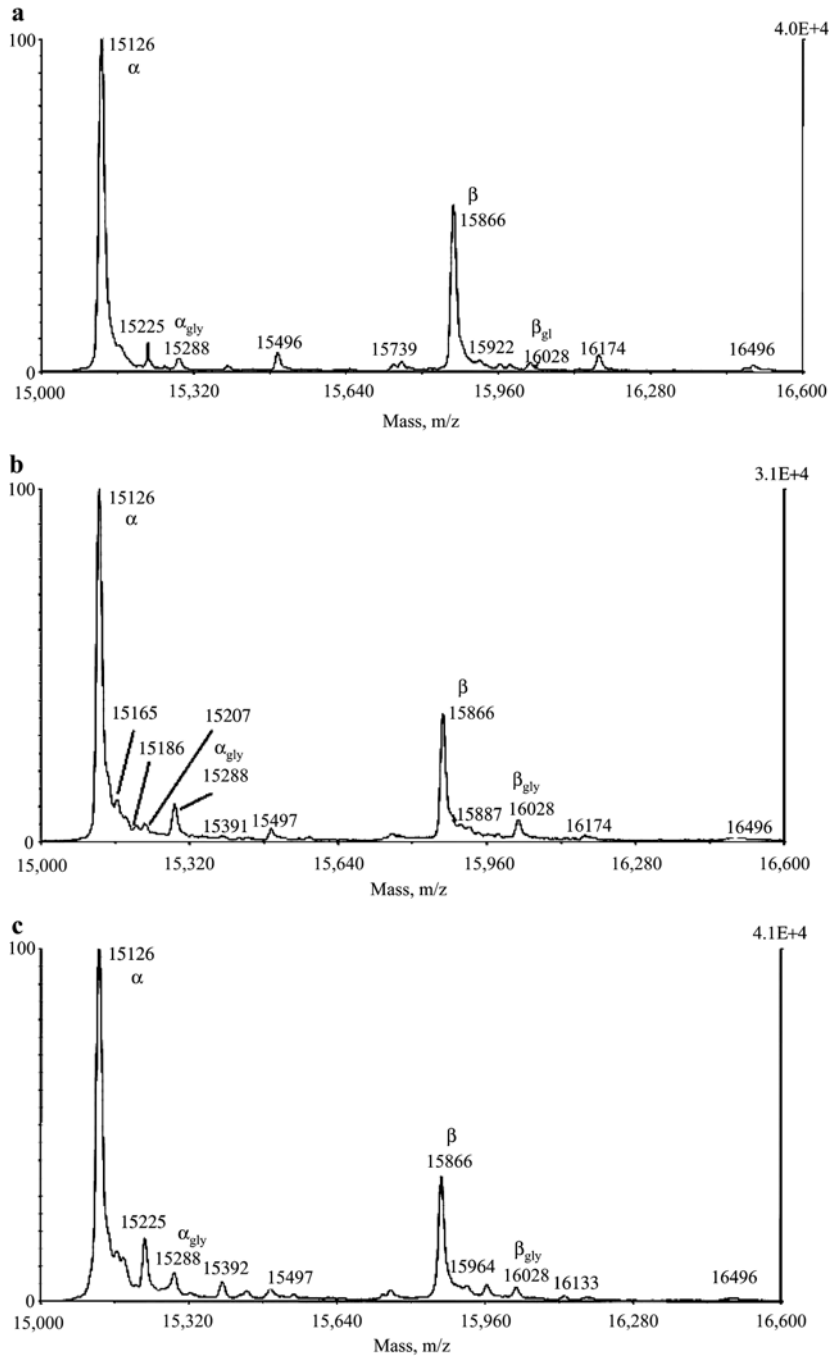

Figure 4 Typical MALDI spectra of the globin fraction in: a normal subject (a); a diabetic patient without complications (b); and a diabetic patient with complications (c).
(Reprinted from Lapolla A et al. The complexity of non-enzymatic glycation product sets of human globins. Diabetologia 1997;47:613–7. Reproduced with the permission of Springer-Verlag.)

by considering the sum of the percentages of the whole pool of glycated and glyco-oxidated α- and β-globins. The resulting plot (Figure 5c) shows that HbA_{1c} correlates more closely with the whole set of glycated and glyco-oxidated products of both α- and β-globins, and not with the simply glycated β-globins, as is usually believed (46–48). Another MS technique was used successfully in globin investigations and proposed as an effective method for quantifying glyco-hemoglobin. This approach was based on ESI mass spectrometry and produced spectra from whole blood samples composed primarily of glycated and unglycated α- and β-globins (49). It was applied to a large number of patients with differing

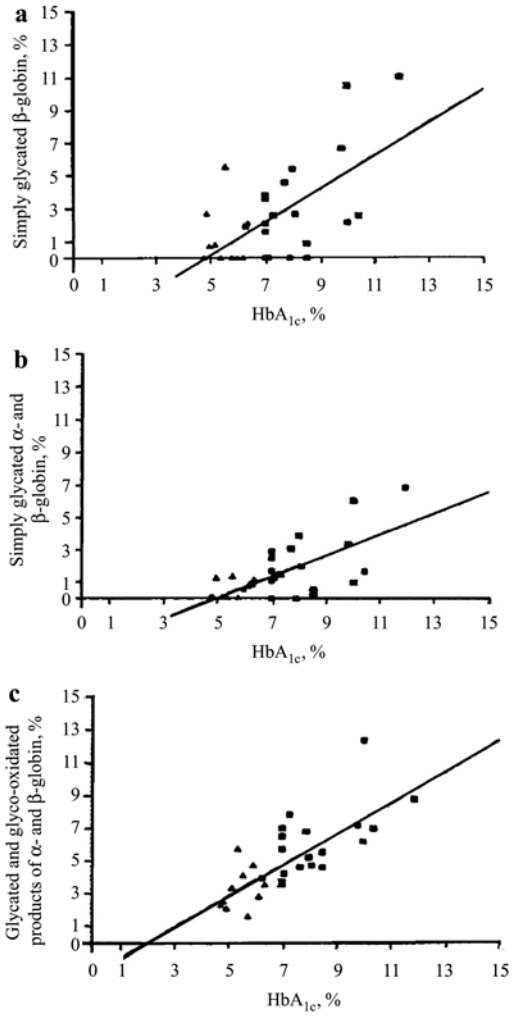

Figure 5 Plot of percentages of: (a) simply glycated β-globins obtained by MALDI vs. HbA_{1c}; (b) simply glycated α- and β-globins obtained by MALDI vs. HbA_{1c}; and (c) whole pool of glycated and glyco-oxidated products of α- and β-globins obtained by MALDI vs. HbA_{1c}. ■, diabetic subjects; ▲, healthy subjects.
(Reprinted from Lapolla A et al. A highly specific method for the characterization of glycation and glyco-oxidation products of globins. Rapid Commun Mass Spectrom 1997;11:613–7. © John Wiley & Sons Ltd. Reproduced with permission.)

degrees of metabolic control, and the quantitative results were linearly consistent with HbA_{1c} measurements.

Applying MS to glycated globin evaluation has the merit of demonstrating that both globins are glycated to a similar extent, contrary to what is commonly believed. MALDI can also identify glyco-oxidated products. These results are consistent with the data reported by Hempe and co-workers (50), indicating the existence of high- and low-hemoglobin glycation phenotypes. However, our results indicate that different subjects have a different proclivity for oxidation processes occurring after globin glycation.

The possible relationship between the MALDI data and the clinical conditions of patients was studied. Interestingly, the presence or absence of chronic complications affected the slope of linear regression in different ways (51). Next, our attention turned to the differences observed between patients with and without complications, due to a different individual proclivity for oxidation and/or differing oxidation kinetics related to behavioral and/or environmental factors. A precise description at the molecular level of the whole set of unglycated, glycated and glyco-oxidated α- and β-globins would be a valuable contribution to the accurate analysis of the different phenotypes, and important for the definition of appropriate pharmacological treatments.

The "classic" proteomic approach

The results obtained in the case of globins, HSA and globulins made us confident of the validity of our analytical approach, which was consequently applied to more complex pathological situations.

Data from epidemiological studies have shown that high-levels of high-density lipoproteins (HDL) in plasma protect against the development of atherosclerosis (52), due to the known actions of HDL (53). In this context, apolipoprotein A-I (apoA-I) which constitutes 70% of the apolipoprotein content of HDL, acts as an acceptor for the transfer of phospholipids and free cholesterol from peripheral tissues, and transports cholesterol to the liver and other tissues for excretion and steroidogenesis (54). Atherosclerotic vascular disease is one of the major complications of diabetes and end-stage renal diseases (55–57).

In a preliminary investigation, a proteomic approach was employed to study plasma samples from healthy, diabetic and

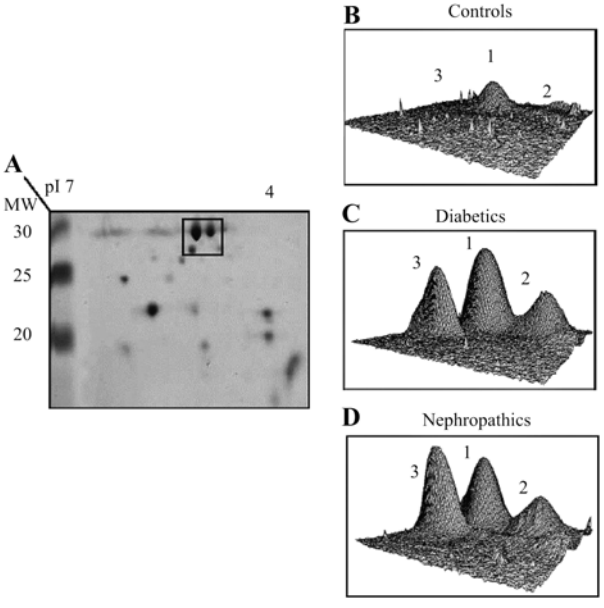

Figure 6 Analysis of the three group of samples by 2-DE.
(A) 2D plots of 2-DE protein spots. 3D views of the area of interest of: (B) controls, (C) diabetics, and (D) nephropathic subjects. (Reprinted from Lapolla A et al. On the search for glycated lipoprotein ApoA-I in the plasma of diabetic and nephropathic patients. J Mass Spectrom 2008;43:74–81. © John Wiley & Sons Ltd. Reproduced with permission.)

nephropathic subjects to identify possible post-translational modifications to apoA-I due to non-enzymatic glycation processes. MALDI was used to identify the protein of interest by peptide mass fingerprinting (PMF) after enzymatic digestion (58). The sequences of modified amino acids were obtained by means of post-source decay (PSD) (59) experiments.

The results of the 2-DE analysis on the three groups of samples are shown in Figure 6. Significant differences between the three groups are clearly visible in the 3D views of the area of interest (Figure 6, panels B–D). Although there is essentially only one peak in the 3D plot for the healthy subjects, three different peaks are clearly detectable in the same region in patients with diabetics and nephropathices.

Enzymatic digestion of the differentially expressed spots, followed by MALDI analysis (Figure 7), showed that spots 1 and 2 corresponded to ApoA-I, and spot 3 to retinal binding protein (RBP). This indicated significant overexpression of these proteins in the pathological cases examined. In particular, enzymatic digestion of spot 2 followed by MALDI analysis showed that this protein corresponds to glycated ApoA-I, which was also found in the case of healthy subjects, but to a much smaller extent. The modified peptide sequences were confirmed using the PSD approach, followed by peptide sequence tagging (PST) to filter the database (58, 60, 61).

These findings imply that glycated apoA-I is as abundant as unglycated protein in the plasma from both diabetic patients and patients with nephropathy. Considering the same amounts of plasma, both unglycated and glycated proteins were overexpressed in these two groups by comparison with the healthy control subjects.

Glycated apoA-I concentrations can thus be correlated with the glyco-oxidation stress experienced by patients during the protein's half-life, and the change in protein functionality due to glycation reflects different efficiency in cholesterol transport. This aspect could help explain diabetic cardiovascular disease. It should be emphasized that the same trend is also seen in patients with end-stage renal disease, but this is due to a different mechanism, related to the efficiency of clearance of glycated ApoA-I. This finding also explains the occurrence of macrovascular disease in both types of patient.

Identification of degradation products of glycated, oxidized, and nitrated proteins The Amadori product produced by the Maillard reaction undergoes a series of oxidation rearrangements and degradations, leading to the formation of a heterogeneous group of substances that are usually called ''advanced glycation end products'' (AGEs) (62). Although their chemical nature has yet to be completely elucidated, we consider Thornalley's proposal (2003) (63)

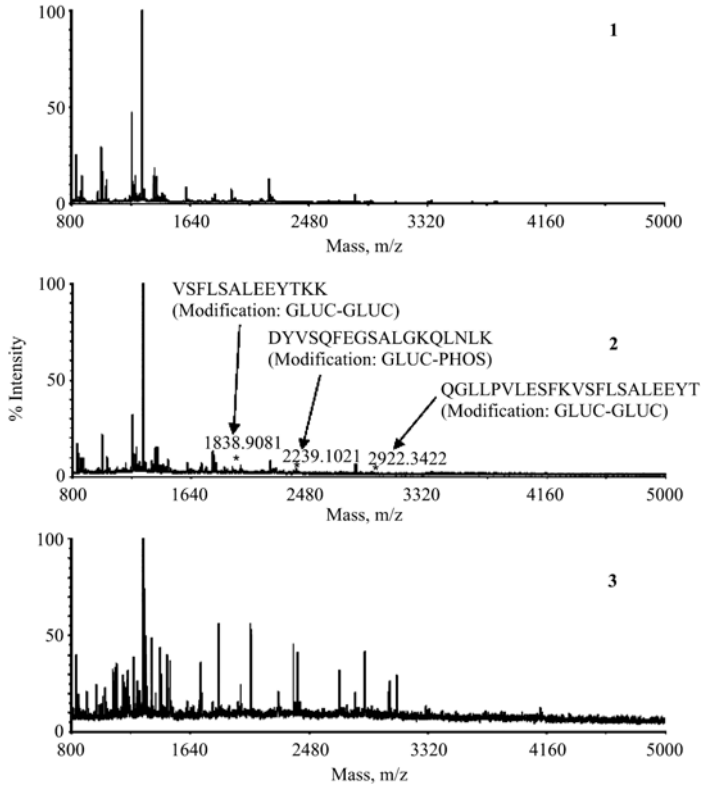

Figure 7 MALDI spectra of the tryptic digested proteins corresponding to spots 1, 2 and 3, differently expressed in control, diabetic and nephropathic subjects, respectively.

(Reprinted from Lapolla A et al. On the search for glycated lipoprotein ApoA-I in the plasma of diabetic and nephropathic patients. J Mass Spectrom 2008;43:74–81. © John Wiley & Sons Ltd. Reproduced with permission.)

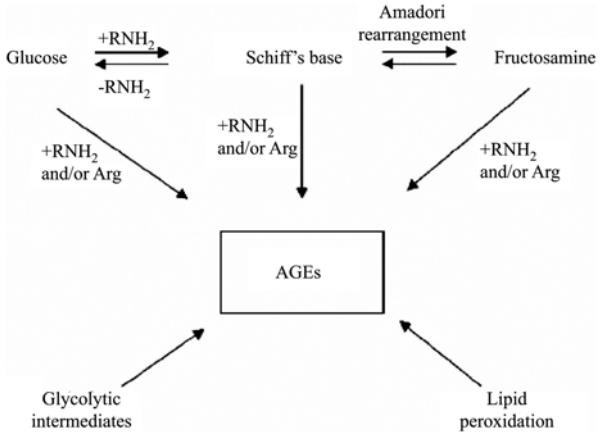

Figure 8 Pathways for the formation of AGEs.
(Reprinted from Thornalley et al. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 2003;375:581–92. © The Biochemical Society. Reproduced with permission.)

the most reasonable. As shown in Figure 8, AGEs can be considered as glycation adducts and may originate via different pathways, mainly involving reactions with glucose, reactive α-oxaldehydes, and other saccharide derivatives.

Reaction with glucose may be particularly relevant in the case of diabetes, because of the higher concentrations of plasma glucose, as well as glyoxal, methylglyoxal, and deoxyglucosones (64, 65).

Figure 9 shows the structural formulas for the α-oxaldehyde glycating agents, early glycation adducts, and AGEs. It is worth noting that they include cross-linking compounds (GOLD, MOLD, DOLD, and pentosidine), which from the physiological point of view represent possible structural modifications of tissue proteins, leading to morphological tissue changes.

Tandem mass spectrometry has been used for the quantitative screening of AGEs in cellular and extra-cellular proteins. The hydroimidazolones were found to be the most abundant glycation biomarkers, while the other AGEs occurred in moderate amounts.

When cells are exposed to high glucose concentrations (5–30 mM), AGEs take approximately 1 week to form, and the related damage can be summarized as follows: (i) intracellular proteins modified by AGEs exhibit altered function; (ii) changes take place in extracellular matrix proteins and cellular integrins; and (iii) plasma protein modified by AGEs gives rise to ligands that bind to the AGE receptor, leading to production of reactive oxygen species, activation of transcription factor NF-Kb, and pathological changes in gene expression in several types of cell (e.g., mesangial and endothelial cells, and macrophages).

An assay has been developed for quantifying AGEs by derivatization and use of a liquid chromatography assay (66). It is based on enzymatic hydrolysis of the protein substrate, derivatization of the hydrolysate with 6-amino quinolyl-N-hydroxy succinimidyl carbamate (AQC), and liquid chromatorgraphy analysis with fluorometric detection.

Structural isomers of methylglyoxal-derived hydroimidazolone, glyoxal-derived hydroimidazolone, 3-deoxyglucosone-derived hydroimidazolone, and N_{γ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2yl)-ornithine concentrations have been determined. The assay has been applied to the characterization of HSA modified by N_{ε}-carboxy-methyl-lysine and N_{ε}-(1-carboxyethyl)-lysine.

A method based on liquid chromatography-tandem mass spectrometry (LC/MS/MS) was recently proposed for the quantitative measurement of 16 biomarkers indicative of protein glycation, oxidation and nitration damage (67). The related investigation showed that, while hydroimidazolones are the most important glycation biomarkers, methionine sulfoxide is the most important oxidative biomarker. For nitration, 3-nitrotyrosine was found to be an effective marker. This study showed that advanced glycation leads to significant changes in cellular and extra-cellular proteins. In addition, the enzymatic defenses against glycation, antioxidant and proteosomal protein degradation inside cells have been indicated as the factors likely to regulate biomarker levels of cellular proteins.

It would be of interest to determine the glycation, oxidation, and nitration reaction sites on the protein in order to gain information on pathological states and hypothesize changes in protein functionality. In this frame, Thornalley's group conducted numerous investigations on both plasma and urine proteins (67–69) the results of which can be summarized as follows.

i. Protein glycation, oxidation, and nitration occur in the presence of high glucose concentrations. The digestion of plasma proteins from diabetic patients enables the identification and quantification of protein residues diagnostic for the reaction that has occurred; the concentrations of these protein residues have been found higher in diabetic patients than in controls.
ii. The glycated, oxidized, and nitrated proteins are enzymatically digested at the systemic level. Thus, free adducts are produced and transported by the plasma and excreted by the kidney. Their concentrations have been evaluated and found to be higher than in undigested proteins.

Glycation-free adducts have a high renal clearance that declines markedly in the case of acute and chronic renal failure, giving rise to an accumulation in the plasma where they act as a uremic toxins (68–70).

Urinary protein profile

Nephropathy is one of the most important long-term complications of diabetes, leading to kidney impairment and in some cases to end-stage renal disease (71).

It is important from a diagnostic point of view to monitor any potential kidney impairment at the molecular level, using effective markers for promptly identifying the onset of the disease to allow implementation of suitable therapeutic measures. Urine albumin concentrations are usually considered in this context (72), but the development of further con-

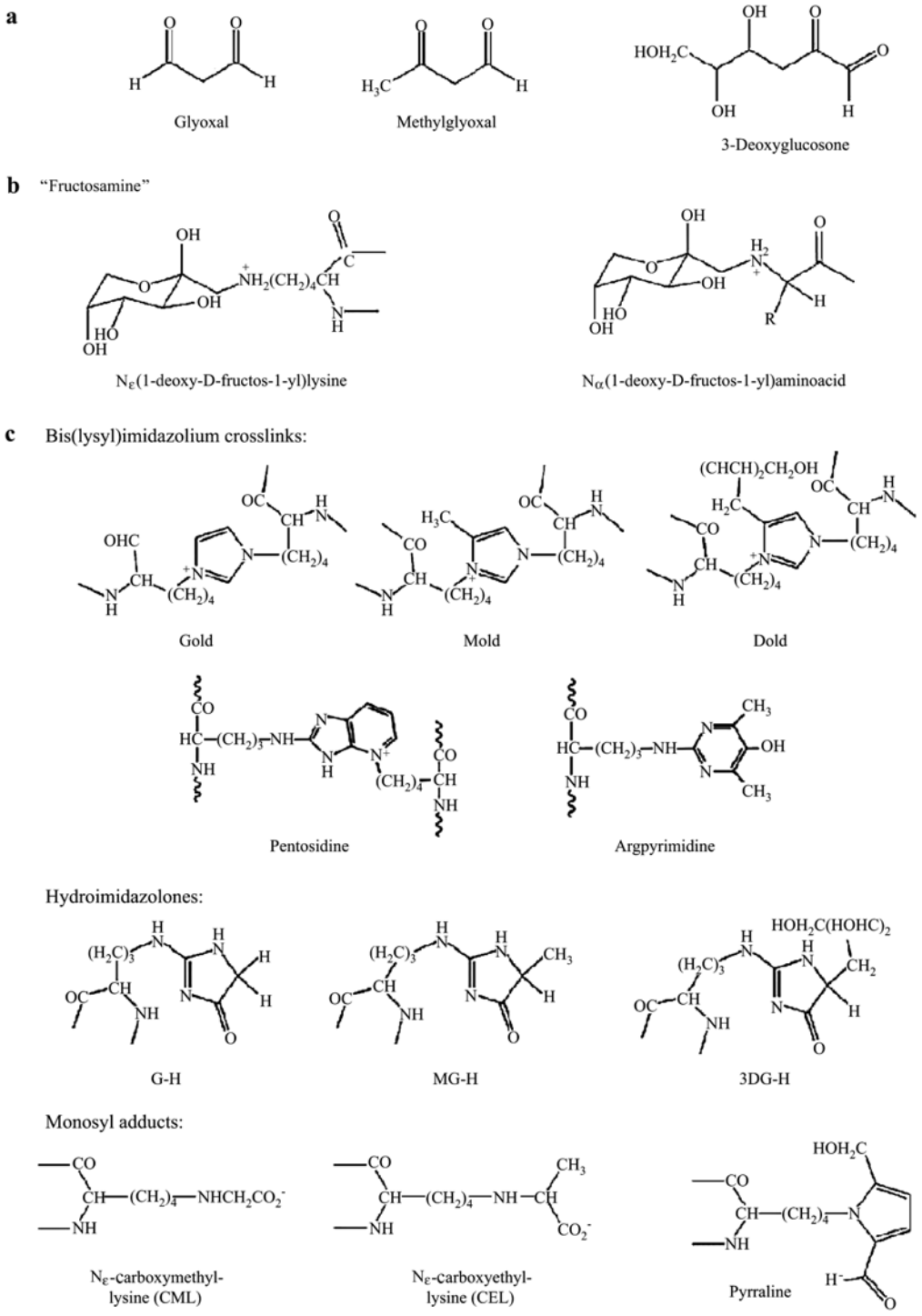

Figure 9 A-oxoaldehyde glycated agents (a); early glycation adducts (b); AGEs (c).
(Reprinted from Thornalley et al. Quantitative screening of advanced glycation end-products in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 2003;375:581–92. © The Biochemical Society. Reproduced with permission.)

firmatory assays is definitely of interest. Rao et al. recently performed an extensive study using a proteomic approach to identify possible biomarkers of diabetic nephropathy (73). Using differential in-gel electrophoresis (DIGE) followed by tryptic peptide analysis with LC/MS/MS, the author identified seven proteins that were upregulated and four proteins that were downregulated with increasing albuminuria.

During the same period, an extensive investigation was performed by Mischak et al. (74), devoted primarily to assessing diabetic renal damage in humans in terms of the

urinary protein profile. Using a highly specific method based on CZE coupled with ESI/MS, the authors identified a ''normal'' urinary polypeptide pattern in 39 healthy subjects that clearly differed from the pattern seen in 112 patients with type 2 diabetes mellitus. This led to the design of a specific ''diabetic'' pattern of polypeptide excretion. This approach enabled the detection of peptides indicative of diabetic renal damage in patients with high urine albumin concentrations.

The same approach (CZE/ESI/MS) was used to identify the urine protein patterns in type 1 diabetic adolescents with early diabetic nephropathy (75). Among more than 1000 different polypeptides (in the mass range of 800–66,500 Da), the method revealed a specific cluster of 54 polypeptides found only in the urine of diabetic patients.

In another study, CZE/MS analysis was used to evaluate the changes in urinary polypeptide patterns during treatment with the angiotensin II receptor blocker (ARB), candesartan (76). Treatment with this compound significantly changed 15 of the 113 polypeptides characteristic of patients with macroalbuminuria, suggesting that this analytical approach can be useful for monitoring the efficacy of pharmacological treatments.

SELDI was used to develop a method for predicting diabetic nephropathy (77). Urine samples from 31 type 2 diabetic patients were analyzed systematically over a 10-year period, correlating changes in the urinary protein profiles with any onset of diabetic nephropathy. The SELDI data were considered as a fingerprint of an individual's physiological/pathological status and the differences were observed from a morphological point of view, without considering protein structure. This method showed that urinary proteomic profiling by SELDI can identify normoalbuminuric subjects with type 2 diabetes who subsequently develop diabetic nephropathy.

CZE coupled with ESI/MS was also used recently by Rossing et al. (78) to elucidate the urinary proteomics in diabetes and chronic kidney disease (CKD). By studying 305 subjects, a panel of 40 biomarkers enabled diabetic patients to be distinguished from healthy subjects with 89% sensitivity and 91% specificity. The characterization of cases of diabetic nephropathy vs. other CKDs reached 81% sensitivity and 91% specificity. Many of the biomarkers identified were fragments of collagen type 1 and their quantities clearly decreased in patients with diabetes or diabetic nephropathy. The uromodulin fragment 589–607 was also detected.

Both the classic proteomics approach and CZE-based measurements are highly-specific methods, but have the drawback of being difficult to use in clinical chemistry laboratories; both involve lengthy analysis times and demand experienced personnel and expensive equipment. Alternative analytical approaches would therefore certainly be of interest. This prompted us to test the power of MALDI/MS in describing the urine protein profile, with a view towards differentiating between healthy, diabetic, diabetic-nephropathic, and nephropathic subjects.

The results of a preliminary study (79) on urine samples from type 2 diabetics, patients with renal disease, diabetics with renal disease and healthy controls showed that the MALDI spectra of the low-molecular-weight fraction of the peptides in urine samples exhibited marked variability within each group, related primarily to their relative abundance. Some differences between the four populations that were examined were clearly apparent, however, on comparing the spectra for samples belonging to the different groups (see Figure 10). It was evident that the spectra for diabetic patients and healthy subjects were very similar: in both cases, the most abundant peak was at m/z 1912, while other, less abundant peaks were seen in both cases at m/z 1219 and 2049. This last species was underexpressed in samples from diabetic patients. The peaks characteristic of the samples from healthy subjects and diabetics were much lower in those with nephropathy and diabetic-nephropathy, whose most abundant peak was at m/z 1219, whereas the ion at m/z 1912 was strongly suppressed. These data are shown in Figure 11.

The MS/MS spectrum of the ion at m/z 2049 was identical for all the samples examined and the search using the Protein-Pilot v.2.1 software in the Uniprot Database indicated that its sequence is NGDDGEAGKPGRHypGERGPHypGP, corresponding to the collagen α-1 (I) chain precursor (80).

Using the same approach, the ion at m/z 1912 was found to have the sequence SGSVIDQSRVLNLGPITR, and to correspond to the uromodulin precursor.

The ion at m/z 1219 was found due to the IGPHypGP-HypGLMGPP sequence in the collagen α-5 (IV) chain precursor.

The under-expression of the uromodulin fragment seen for nephropathic patients with advanced renal disease and diabetic patients with advanced nephropathy might be related to alteration of the apical cell membrane of the thick ascen-

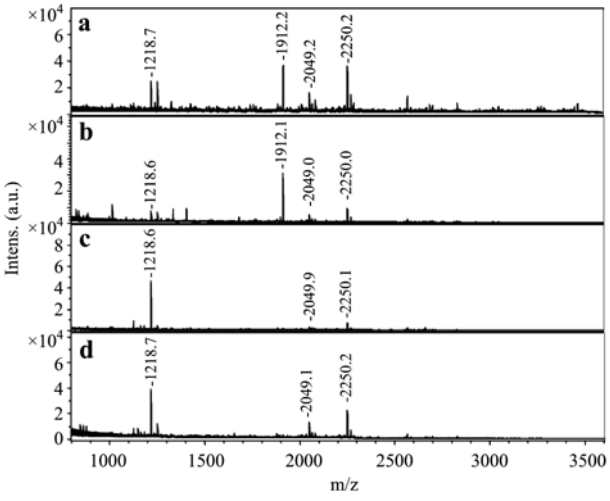

Figure 10 MALDI mass spectra of urine samples.
From: (a) control subject; (b) diabetic patient; (c) nephropathic patient; and (d) diabetic-nephropathic patient. (Reprinted from Lapolla A et al. Low molecular weight proteins in urine from healthy subjects as well as diabetic, nephropathic and diabetic-nephropathic patients: a MALDI study. J Mass Spectrom 2009;44:419–25. © John Wiley & Sons Ltd. Reproduced with permission.)

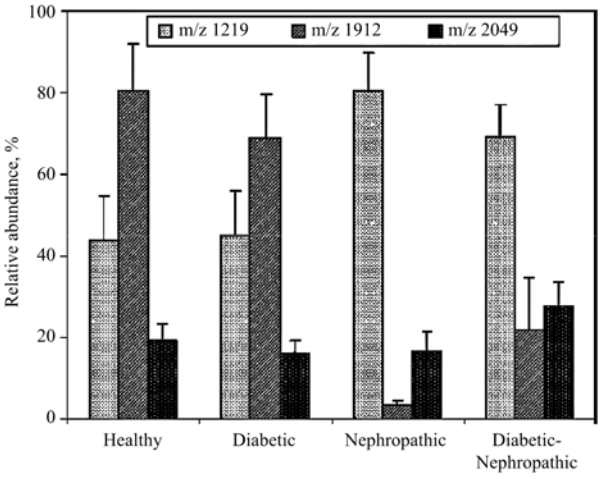

Figure 11 Histograms of the abundance of ions at m/z 1219, 1912 and 2049 found in urine samples from the subjects under study. Data are expressed as mean ± SEM. (Reprinted from Lapolla A et al. Low molecular weight proteins in urine from healthy subjects as well as diabetic, nephropathic and diabetic-nephropathic patients: a MALDI study. J Mass Spectrom 2009;44:419–25. © John Wiley & Sons Ltd. Reproduced with permission.)

dant limb (TAL) epithelial cells, while the collagen fragments could be explained by an alteration of the renal basal glomerular membrane. Further investigations on a larger population confirmed these findings.

Conclusions and possible future trends

Applied to proteomics, the latest mass spectrometric techniques have proved highly effective in shedding new light on the molecular changes induced by diabetes.

The high glucose concentrations in diabetic patients lead to protein glycation, with consequent changes in protein functionality. Mass spectrometry enables the number of glucose molecules condensed on the protein to be determined, and also the glycation sites after enzymatic digestion. This provides physicians with a new diagnostic tool for diabetes control, as well as a more complete view of the pathology of diabetes.

Some studies have demonstrated the power of mass spectrometry, not only in glycation studies, but also in elucidating protein modifications due to oxidation and nitration, which correlate well with the development of pathological states.

A broader array of mass spectrometry applications can be expected in the near future, as a result of a general strategy based on investigating highly specific (and very costly!) technologies to identify valid markers of the disease's evolution, and the subsequent development of more straightforward instrumental approaches for use within clinical chemistry laboratories. This will reflect on the current availability of powerful methods for diabetes management.

In the more distant future, if the current trends continue we can expect new technologies to be developed with higher sensitivity and wider dynamic ranges, with positive implications for the medical world.

In this scenario, the use of a method capable of providing information at the tissue level seems to be highly promising. Desorption electrospray ionization (DESI) could provide a picture of the low-molecular-weight molecular species while ion imaging by MALDI could, due to its spatial resolution, give us information on the distribution on the tissue of different molecules of interest (large and small, such as proteins and peptides).

Conflict of interest statement

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