Emerging biotechnological approaches with respect to tissue regeneration: from improving biomaterial incorporation to comprehensive omics monitoring

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4.1 Introduction

The development of biomaterials has demonstrated an unprecedented progress which has been emphasized by the introduction of a significant number of innovative materials. As such, the advent of biomaterials represents a formidable opportunity for the implementation of therapeutic treatments, able to induce tissue or organ regeneration [1]. Meanwhile, improved understanding of complex biological mechanisms and the enhancement of the production process of biological (macro)-molecules using state-of-the-art biotechnologies have also experienced some major breakthroughs [2−4]. These progresses have led to their wide adoption for therapeutic purpose. Indeed, different types of proteins, such as monoclonal antibodies (mAbs) or fusion proteins, are currently approved for therapeutic use and the first biopharmaceutical product based on siRNA has been approved by the FDA on the third semester 2018 [5−8]. As a consequence, recent research articles describe the incorporation of biomolecules such as proteins to the structure of biomaterials in order, for instance, to deliver locally biological agents capable of favoring the regeneration of the tissue or improve the integration of the biomaterial [9,10].

Due to their inherent structural complexity and crucial implications, the incorporation of biomolecules to the material requires a dedicated characterization. The analysis of highly complex macromolecules, especially in complex matrices such as biological samples, is used to represent a challenge for the analytical sciences.

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A deeper understanding of the structure of biological macromolecules and the introduction of innovative instrumentation and methodologies, such as, for instance, -omics analysis, have allowed to alleviate the limitations of conventional analytical methodologies. Still, the characterization of biomolecules requires to be based on a multilevel analysis relying on complementary analytical methodologies, which provide different types of information. This chapter is focusing on providing insights on various cutting-edge analytical methodologies, such as high-resolution mass spectrometry (MS). These different analytical techniques can be implemented in order to study, on one hand, the influence of biomaterials incorporation to the structure of the biomolecules and on the other hand, to monitor the outcome of the therapeutic agent.

4.2 Analytical methodologies for protein identification and monitoring

In the context of the development of innovative biomaterials for tissue regeneration, the need for protein identification and/or monitoring arises from several aspects. Because of its exogenous origin, the in vivo presence of a biomaterial involves some major consequences especially on a systemic level [11]. As an illustration, the impact on the immune system is most prominent mainly due to its natural protecting function against foreign bodies [12]. A significant number of research articles are discussing the immunological reactions triggered by the exposure of biomaterials [13,14]. The biological processes involved in the immune response are regulated by the expression of different proteins such as tumor necrosis factor-α [15,16]. As a consequence, the possibility to identify in a specific manner this type of proteins and monitor their respective levels of expression can provide decisive information regarding the systemic impact of a biomaterial. The monitoring of various protein markers has been used to understand in a comprehensive way the implication of the biomaterials on the immune response in order to develop materials which could generate a reduced response and minimize the occurrence of side effects [17].

From another perspective a significant number of biomaterials recently developed for tissue regeneration rely on the incorporation of proteins to the structure of the biomaterial. Among applications the incorporation of proteins to the structure of biomaterials can be urged by further functionalization of the material [18], improved compatibility with the microenvironment [19], or controlled modulation of the immune response [10]. Thus in the most advanced applications, the biomaterial is gradually releasing the protein in order to generate the desired effect. It is therefore necessary to have analytical techniques capable of providing specific detection and absolute quantification of the incorporated proteins, in order to assess the material for the protein release capacity in addition to providing consistent data on the release profile.

Regardless of the investigated aspect, the analytical methodologies require to provide an outstanding specificity in order to give the detection of a single protein present in complex biological samples for which available volume are commonly low. Similarly, the used techniques have to be particularly sensitive due to the low concentration of proteins generally present or released, which are analyzed in mixture with a large number of proteins, generally exhibiting significantly higher concentrations. Finally, in such a demanding context, the analytical strategies employed need to provide the analysis of several protein markers simultaneously. Over the last three decades a few analytical techniques have emerged and demonstrated to be particularly adapted to this type of analysis. As such, the major instrumental developments of flow cytometry (FC) have demonstrated its relevance to investigate in a comprehensive manner the immune response.

MS is an analytical technique that enables the detection, the identification, and the quantification of the compounds constituting a sample by measuring their *m/z* ratio. The fundamental principle of MS consists of generating, from the studied molecule, ions in the gas phase by the intermediate of an ionization source. The ions produced are then separated based on their mass-to-charge (*m/z*) ratio and detected independently. Because the relation between the physical phenomenon used to separate the ions and the *m/z* ratio is known; it is possible to deduce the *m/z* ratio of an unknown molecule and, therefore, determine with an excellent accuracy its molecular mass. MS instruments can be virtually partitioned in four distinctive parts: the ionization source participates to the ionization and emissions of the compounds in the gas phase, the transfer line ensures the transport of the formed ions to the mass analyzer, the mass analyzer separates the compound in order to determine their *m/z* ratio, and the detector which produces an electrical signal handled by the data-treatment system (Fig. 4.1). MS is characterized by an outstanding specificity which allows to distinguish compounds exhibiting minor *m/z* ratio differences. In addition, MS is a particularly sensitive technique, with contemporary MS instruments able to detect compounds at subpicomolar (10^{-12} mol/L) level. At the same time, the different types of MS instruments available make it compatible with the analysis of a large variety of molecules. Finally, the use of dedicated approaches, such as tandem MS (MS/MS), can allow to obtain to some extend further structural information regarding the analytes. Thereby, more than a simple detection method, MS is considered today as one of the major analytical techniques. Although is a centenary technique, MS analysis of polar molecules such as biomolecules was made possible relatively recently by the development of compatible ionization sources, electrospray ionization (ESI), and matrix-assisted laser desorption

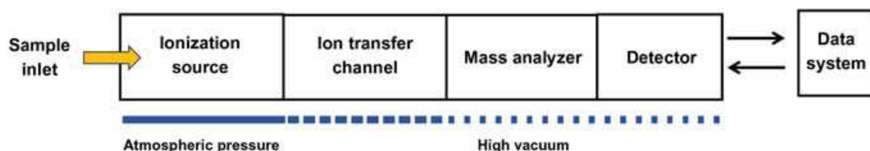

Figure 4.1 Schematic representation of the different sections constituting MS instruments.

ionization (MALDI). However, only introduced at the end of the 1980s, MS has demonstrated to be an outstanding tool for the analysis of a wide variety of biomolecules such as peptides and proteins.

Commonly, a separation is often implemented prior to the MS analysis. Even though MS instruments are capable of detecting several compounds simultaneously and determine their respective *m/z* ratio if significantly different, the use of a separation method possesses different positive implications. Indeed, the preliminary fractionation of the compounds constituting a sample and their progressive transfer to the MS instrument is particularly relevant for the analysis of complex samples, giving the possibility to improve the number of compounds effectively detected and lowering the competition effect potentially occurring during the ionization process [20]. Liquid chromatography (LC) coupled with MS (LC–MS) is therefore used in most of the cases for the analysis of biological molecules such as nucleic acids, peptides, and proteins. When performing LC separation, the sample is carried inside a stationary phase by the intermediate of a constant flow of liquid phase referred to as mobile phase. The flow of mobile phase is created by a set of pumps able to deliver a flow rate accurately adjusted and extremely constant, even at high pressure in order to achieve efficient and reproducible separations. The stationary phase is constituted by a porous solid, densely packed in order to form a chromatographic column. Consequently to their introduction to the stationary phase, the different compounds constituting the sample are experiencing retention to the stationary phase. Because the retention is conditioned by the physicochemical characteristics of the compounds, they will exhibit significantly different retention times. Thus the components of a mixture will be eluted from the chromatographic column at different times and then gradually transferred to the MS instrument. The most common type of chromatographic stationary phase, referred as reverse phase, is composed of silica particles modified with alkyls groups (e.g. C18H37, C8H17) in order to make the media hydrophobic and apolar. However, various types of stationary phases are available in order to adapt the retention, which offers the possibility to change the selectivity of the separation. From an instrumental standing point, LC separation is realized on a routine basis mainly using high-performance LC (HPLC) instruments. Typically, HPLC is implementing stationary phases with particle size from 2 to 10 μm and a flow rate commonly comprised between 0.5 and 1.5 mL/min. More recently, miniaturized LC instruments, referred to as nanoLC, have been developed for routine analysis. Regardless of the format, LC–MS hyphenation can be quite straightforward, especially for ESI–MS instruments. Indeed, the mobile phase outlet can be directly connected to the sample introduction component of the MS system. Therefore the principal requirement is that the mobile phase is compatible with MS analysis and sufficiently volatile to be eliminated in the ESI source. Especially, it is the case for the mobile phases composed of H_2O/ACN or H_2O/MeOH mixtures, typically used in reverse-phase chromatography.

The advent of MS analysis applied to biological molecules has opened new horizons for the (bio)analytical chemistry field. Consequently, LC and MS instruments have benefited from major technical improvements which have allowed to constantly improve the sensitivity and level of characterization achieved using LC–MS

analysis. The performances of contemporary LC–MS instrumentations are now capable of providing a tremendous amount of data in a single LC–MS analysis which gives the possibility to achieve in-depth characterization of highly complex biological samples enabling the emergence of MS-based proteomic analysis.

4.3 Mass spectrometry–based proteomic analysis

The terminology *proteome* designates a complete set of proteins present in an organism or in a compartment (e.g., mitochondria and membrane), at a given time and for a given physiological state. The analysis of the proteome, commonly mentioned as proteomic analysis, consists of the systematic identification composing a biological sample which can be completed with quantitative information. This analytical workflow has been developed consequently to large-scale genomics in order to provide a detailed analysis regarding the diversity of the proteins [21] in an extended manner. Indeed, proteins are resulting from the transcription of the DNA-coded information. However, the evolution of the protein expression levels, depending for instance on the state, is not enclosed in the genetic heritage. It is then necessary to perform the analysis on the protein level in order to attribute minute changes in the expression levels. Similarly, proteins are commonly experiencing endogenous chemical modifications described as posttranslational modifications (PTMs) [22]. Notably, some modifications have been attributed to be important in biological signaling like deamidation [23,24] or phosphorylation [25]. In this case as well, genomic analysis does not provide information regarding that aspect therefore requiring a direct characterization of the protein.

Originally, proteomic analyses were mainly performed using bidimensional differential gel electrophoresis (2D-DIGE). Due to their chemical nature, proteins are widely diverse in terms of size and they also bear a significant number of charged functions in solution. As a consequence, the separation provided by gel electrophoresis demonstrated to be particularly adapted to proteins with the possibility to achieve high resolution even for complex biological samples [26]. However, 2D-DIGE requires a specific experimental expertise. Also, this analytical approach does not provide unambiguous identification of the proteins and additional experiments are needed in order to obtain a structural identification of the separated proteins. The introduction of MS instrumentation adapted to the analysis of biomolecules has modified in a drastic manner the proteomic analysis [27]. Indeed, MS possesses the interest to provide an outstanding sensitivity as well as specificity, which is particularly relevant for the analysis of complex samples. In addition, it can be coupled in a straightforward manner to LC in order to implement a separation prior to the detection.

In state-of-the-art proteomic analysis or bottom-up proteomics, the protein mixture composing the sample is undergoing proteolytic digestion using endoprotease enzymes. The complex mixture of peptides is then separated and analyzed by LC hyphenated to MS/MS (LC–MS/MS) as emphasized in Fig. 4.2. The peptides are

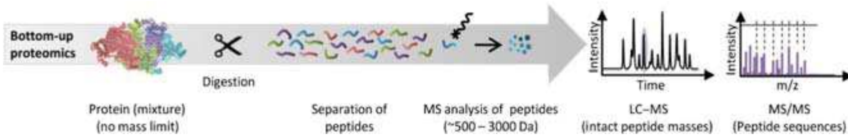

Figure 4.2 Schematic representation of a generic MS-based proteomics experiments, including proteolytic digestion, to generate the peptide mixture which is then analyzed by LC–MS/MS to obtain high-resolution MS measurement of the peptide in conjunction to the fragmentation spectrum. *LC–MS/MS*, Liquid chromatography hyphenated to tandem mass spectrometry; *MS*, mass spectrometry.
Source: Adapted with permission from Switzar L, Giera M, Niessen WMA. Protein digestion: an overview of the available techniques and recent developments. J Proteome Res 2013;12(3):1067–77 [28]. ©2013 American Chemical Society.

then gradually transferred to the MS instrument where their *m/z* ratio is measured followed by their fragmentation in the gas phase in order to measure the *m/z* of the fragments (Fig. 4.2). The conjunction of these two information is used to identify unambiguously the different peptides by direct homology against a protein sequence database used to generate theoretical peptides in silico by the intermediate of a search algorithm [29–31]. MS instruments have experienced some major improvements over the last two decades, especially regarding to the MS accuracy, resolution, sensitivity, and acquisition frequencies. These improvements have allowed to push further the level of characterization achieved using proteomic analysis. As a direct consequence, it is possible to perform the proteomic analysis on complete protein extracts without any type of prefractionation, which is commonly referred to as shotgun proteomics, just by performing directly a complete digestion of the entire sample content [32]. Therefore the proteomic analysis has over the recent years demonstrated to be particularly powerful with the possibility using state-of-the-art MS instrumentation to identify in a single analysis more than 10,000 proteins using an injected quantity of sample generally corresponding inferior to 1 μg of protein extract [33,34]. Also, this analytical workflow is compatible with the incorporation of quantification elements in order to monitor the evolution of the expression level of the identified proteins [35–37]. The proteomic analysis workflow is partitioned into three consecutive phases which requires critical optimization depending on the sample nature and complexity to enable a relevant characterization: sample preparation, (nano)LC–MS/MS analysis of the peptide mixture, and finally data treatment.

4.3.1 Sample preparation for proteomics experiments

Proteomic analysis is based on peptide characterization in order to lead to the identification of the protein composing the sample. From an MS standing point the analysis of peptides is more favorable due to significantly higher ionization efficiencies of the ESI source interfacing the LC system and the MS system, compared to intact proteins. Also, because of their reduced size, the fragmentation of peptides requires

less energy therefore yielding an important number of specific fragments, which enables a systematic and high-fidelity peptide identification from fragmentation spectra.

To generate the peptide mixture from samples mainly composed of proteins, the sample preparation designed for proteomic sample relies on proteolytic digestion using different types of proteases. Proteases are enzymes whose biological activity involves the cleavage of the peptide backbone of proteins [38]. Prior to proteolytic digestion, proteins are commonly undergoing a reduction process in order to cleave disulfide bridges between cysteine residues and therefore destabilize the tertiary/quaternary structure of the protein. Different reagents can be used to perform the disruption of disulfide bridges such as ß-mercaptoethanol or Tris (2-carboxyethyl) phosphine−HCl [39] however dithiothreitol (DTT) represents by far the most commonly adopted alternative (Fig. 4.3A) [40]. That observation is explained by the efficiency of the reduction, the favorable kinetics allowing the reduction to be realized rapidly in addition to minor interferences with the downstream (nano)LC−MS/MS analysis. Because the reduction is a reversible process, the free thiols are submitted to alkylation in order prevent the reformation of the disulfide bonds [41]. Alkylation can be realized using various types of alkylation agents which covalently bond the thiols present on reduced cysteine residues. One can cite iodoacetic acid or *N*-(2-aminoethyl)maleimide, still the alkylation agent most widely implemented is iodoacetamide (IAM). IAM is a highly reactive alkylation reagent capable of performing the reaction in less than 1 hour in a reproducible manner (Fig. 4.3B) [42,43].

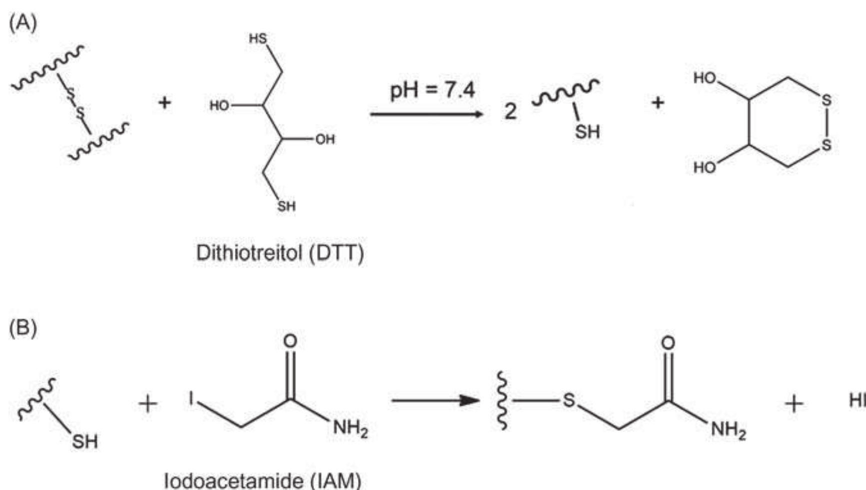

Figure 4.3 Schematic representation of (A) the reaction of the reduction of disulfide bridges between two cysteine residues using DTT and (B) alkylation by the intermediate of IAM. *DTT*, Dithiothreitol; *IAM*, iodoacetamide.

As mentioned, the principal objective of the reduction−alkylation process is to destabilize the structure of the protein for the enzyme to have access to all the cleavage sites present on the protein backbone and therefore maximize the yield of the digestion reaction. The proteolytic enzymes used for digestion in proteomic experiments have the particularity to provide specific digestion of the protein meaning that cleavages occur on specific amino acid residues. That aspect is particularly important to predict in silico the set of peptides generated from the digestion of proteins which enables correlation with the proteins listed in the database. To achieve that purpose, different types of proteases are available exhibiting their respective specificities [43]. Still, trypsin represents the enzyme most commonly used. Trypsin is cleaving specifically the peptide backbone in C-termini of lysine (Lys) and arginine (Arg) residues [44]. Due to the occurrence probabilities of these types of amino acid residues, peptides generated exhibit in the typical case, molecular masses compatible with high resolution MS measurements and concomitant fragmentation in the gas phase to enable MS/MS characterization. In order to provide an optimal activity of the enzyme, the digestion is performed in a dedicated buffer, commonly $(NH_4)HCO_3$ at a concentration of 50 mM (pH 8.0), whereas trypsin is also active at physiological pH but irreversibly inactivated in pH conditions below 4 [45]. The digestion process is then performed by incubation at room temperature for up to 12 hours depending on the nature of the proteins and the complexity of the sample. The reaction can be quenched by addition of trifluoroacetic acid (TFA) or preferably formic acid (FA). Indeed, TFA was determined to be responsible of additional background noise due to its ionization during the ESI process [46]. The sample is finally diluted to the desired concentration using the LC mobile phase.

4.3.2 Peptide mixture analysis by liquid chromatography coupled to tandem mass spectrometry

Consequently, to the digestion of the sample, the mixture of peptide obtained is separated and analyzed by (nano)LC hyphenated to high-resolution MS/MS (nanoLC−MS/MS). Indubitably, the development of nanoLC has been urged by the emergence of proteomic applications. The implementation of LC in a nanometric format allows, on one hand, to achieve high separation efficiencies compared to their analytical counter parts by the intermediate of stationary phases composed of smaller particle sizes [47]. This aspect is interesting with respect to the tremendous complexity of proteomic samples. On the other hand, nanoLC involves the use of lower mobile phase flow rates, typically below 500 nL/min which is particularly favorable to the ESI process, especially due to reduced interferences originating from the mobile phase such as ion suppression effects [48]. It is important to note that nanoLC requires the use of dedicated instrumentation able to deliver accurately controlled and robust nano flow rates. Especially, the pumps used for the circulation of mobile phase require to deliver a flow rate inferior to 500 nL/min in an accurate and constant manner. Therefore conventional HPLC pumps cannot be used anymore. Thereby, higher sensitivity is achieved using nano flow rates [49],

which offers the possibility to achieve a relevant analysis even if the quantity of sample generally available is fairly limited. In addition, this characteristic, supported by the excellent sensitivity of MS instrumentation, allows the identification of lowly abundant proteins [50].

The separation of the peptides composing the digested sample is performed using reverse phase nanoLC basically using a C18-based stationary phase and a mobile phase composed of a mixture of water and acetonitrile containing generally 0.1% FA operated using a conventional gradient [43]. Because of the volatility of its components, this mobile phase system is rather compatible with the ESI process by allowing an efficient elimination of the solvent. The gradient time is adapted to the complexity of the sample; thus gradient time is usually 60 minutes long however it can be extended to several hours in the case of highly complex samples (Fig. 4.4). An extended gradient allows a gradual transfer to the MS of the different peptides composing the sample in order to enhance the number of peptide effectively detected [52].

Following their separation, the peptides are directly transferred to the MS instrument commonly by the intermediate of an ESI source. The instrument is automatically operated in a sequential manner in order to provide the measurement of the *m/z* ratio of the digested peptides (MS analysis) followed by fragmentation in the gas phase, using collision-induced dissociation (CID). The *m/z* values corresponding to the fragments generated from the selected peptide are also measured in a fashion conventionally referred to as MS/MS. CID process relies on the collision of the selected parent ion with inert gas molecules such as N_2 or He to generate the fragmentation. This fragmentation technique has proven to be particularly relevant for the sequencing of peptide, yielding mainly b- and y-ions, in a consistent manner (Fig. 4.5) [54].

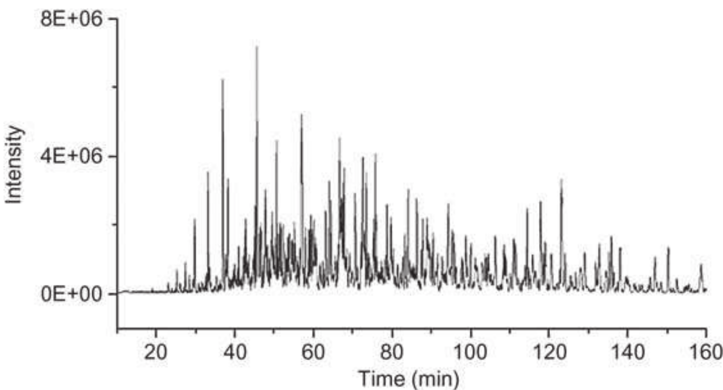

Figure 4.4 Base peak chromatogram achieved from nanoLC–MS/MS analysis of 10 ng tryptic digest of *Shewanella oneidensis*. *nanoLC–MS/MS*, (Nano)liquid chromatography hyphenated to high-resolution tandem mass spectrometry.
Source: Adapted with permission from Zhu Y, Zhao R, Piehowski PD, Moore RJ, Lim S, Orphan VJ, et al. Subnanogram proteomics: impact of LC column selection, MS instrumentation and data analysis strategy on proteome coverage for trace samples. Int J Mass Spectrom 2018;427:4–10 [51]. ©2018 Elsevier.

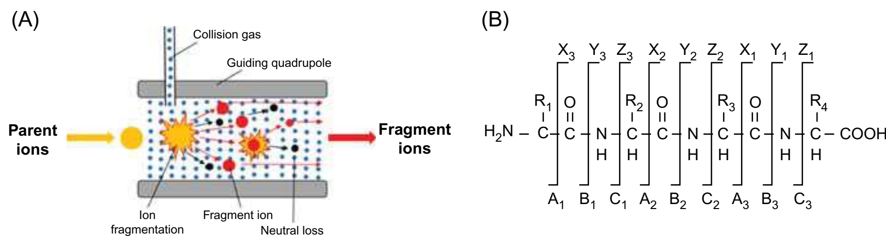

Figure 4.5 (A) schematic representation of a CID collision cell showing the fragmentation performed in MS/MS analysis. (B) Representation of the nomenclature conventionally used to describe peptide fragmentation in MS. Each amino acid is susceptible to generate different fragment ions depending on the cleaved bond mainly driven by the fragmentation mode implemented. *CID*, Collision-induced dissociation; *MS*, mass spectrometry; *MS/MS*, tandem mass spectrometry.
Source: Partially adapted with permission from Roepstorff P, Fohlman J. Letter to the editors. Biomed Mass Spectrom 1984;11(11):601 [53] (panel B). ©1984 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Partially because of the complexity of the sample, the instrument is operated in data-dependent acquisition. Using this mode of acquisition, the instrument automatically detects the elution of a compound based on the intensity of the MS signal, regardless of the *m/z* value measured. Then, the parent ion is selected to undergo subsequent fragmentation in an automatic fashion. This strategy allows to perform the analysis without any a priori knowledge regarding its composition. In addition, the initial *m/z* ratio measured in MS for the parent ion is completely linked to the fragmentation spectra obtained. The analysis is performed using high resolution hybrid MS instrumentation mainly based on quadrupole time-of-flight or orbitrap mass analyzers. These types of instruments are providing high resolution and high mass accuracies and offer the possibility to perform MS and MS/MS experiments (Fig. 4.6). Thus thanks to their high resolution power they are capable of resolving the isotopic profile of complex macromolecules even in the case of peptides of a few kDa (Fig. 4.7A). The plethora of peptides which can be generated from the digestion of the proteins contained in the sample, high resolution MS is required in order to prevent any misidentification. As emphasized in Fig. 4.7, the correlation between the *m/z* measurements of the entire peptide in concomitance with the fragmentation requires to be in complete agreement in order to enable the positive identification of the peptide.

4.3.3 Protein identification

Because of the large amount of data generated from a single experiment, data analysis tends to represent the major bottleneck of the proteomic analysis. To tackle this limitation, different research groups have strengthened their expertise with bioinformatics solutions capable of providing automated identification of proteins

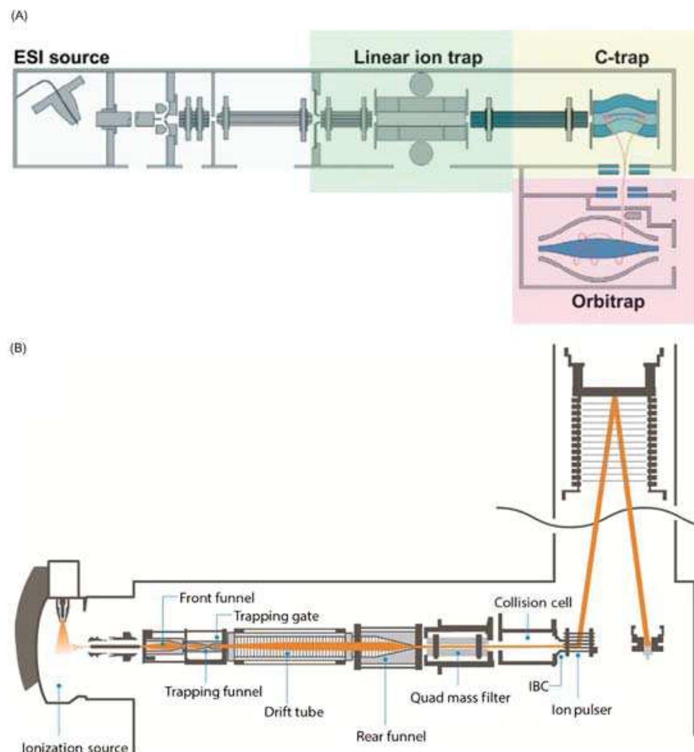

Figure 4.6 Schematic diagram of (A) MS/MS instrumentation based on orbitrap mass analyzer (LTQ orbitrap, Thermo Fischer Scientific) and (B) MS/MS instrumentation based on hybrid quadrupole time-of-flight mass analyzer (Agilent 6560 IM Q-TOF, Agilent Technologies). *MS/MS*, Tandem mass spectrometry.
Source: Adapted with permissions from Scigelova M, Makarov A. Orbitrap mass analyzer − overview and applications in proteomics. Proteomics 2006;6(S2):16−21 [55] and Kurulugama RT, Darland E, Kuhlmann F, Stafford G, Fjeldsted J. Evaluation of drift gas selection in complex sample analyses using a high performance drift tube ion mobility-QTOF mass spectrometer. Analyst 2015;140(20):6834−44 [56]. ©2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim and ©2015 Royal Society of Chemistry, respectively.

using MS/MS datasets and integrate them in a user-friendly workflow. Different solutions are therefore available, either commercial or provided as open-source software [57−60].

Basically, software identification relies in the use of a search algorithm. Using a database composed of proteins amino acid sequence, the algorithm generated in silico a set of theoretical fragmentation spectra depending on the proteins present in the database and also the type of proteolytic enzyme used. This set of spectra is compared to the experimental data obtained from nanoLC−MS/MS experiments. The matching spectra enable the identification of the peptides by homology with the theoretical MS/MS generated from the database. The identification of the

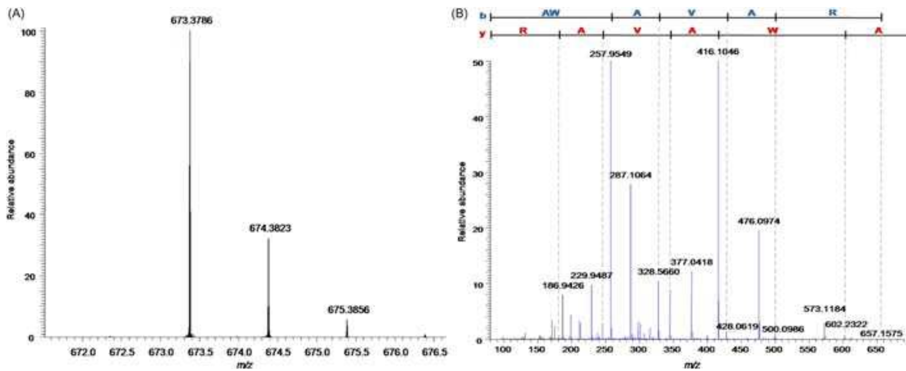

Figure 4.7 Spectra obtained from the Ultra-High Pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) analysis of a tryptic peptide generated from human serum albumin illustrating (A) MS measurement of the peptide correlated with (B) the corresponding MS/MS spectra enabling the identification of the peptide exhibiting the amino acid sequence AWAVAR. *MS*, Mass spectrometry; *MS/MS*, tandem mass spectrometry.

peptides allows to backtrack their origin in the database which ultimately provides the identification of the proteins present in the sample. Thanks to the resolution and mass accuracy provided by high resolution MS, the search algorithm is able to apply harsh restriction, such as the implementation of decoy protein sequences, in order to minimize as much as possible false positives. The search algorithms available also systematically include a scoring system based on different criteria such as the number of fragment identified [61]. The specificity of the peptide identified for the protein is also a key criterion to avoid misinterpretations. Still, the user is able to manually interpret MS/MS data if necessary.

The use of bioinformatics tools has enabled high-throughput and confident identification of proteins using nanoLC−MS/MS large datasets obtained from proteomic experiments. Their large adoption, in conjunction to major instrumental developments, has contributed to explore further extensively complex protein samples. They clearly participated in the establishment and application on a larger scale of proteomic analysis.

4.3.4 Applications of proteomic analysis to the development of biomaterials for tissue regeneration

Proteomic analysis can supply a large amount of information regarding the protein content of samples which may be exploited in different manners. Primarily, data obtained from proteomic experiments can be used to identify the protein diversity composing a biological sample [62,63]. Also, by adaptation of the sample purification, it gives the possibility to focus the investigation on a specific compartment

such as mitochondria or exosomes [64–66]. Historically, this analytical methodology has been used for biomarker research by comparison of different individuals and conditions [67,68]. Quantitative information may also be obtained from experiments in order to interpret changes in protein expression levels [69,70]. The quantitative proteomic approach has been applied for instance to evaluate the influence of environment on various types of organism [71–73].

Conventional MS analysis has been previously applied to the characterization of biomaterials in order to obtain important information regarding their intrinsic structure. As an example, time-of-flight secondary ion MS (TOF-SIMS) has been implemented to perform surface analysis in the form of imaging experiments. Using TOF-SIMS imaging, Belu et al. achieve the precise characterization of the drug metoprolol encapsulated in ethylcellulose. The TOF-SIMS imaging experiments demonstrated the correct encapsulation of the drug, the formation of the ethylcellulose coating in addition to show the silica core of the particles [74]. To provide this type of characterization, the different compounds are previously analyzed independently in order to determine specific *m*/*z* signal they are respectively exhibiting. The biomaterial sample characterized by multiple TOF-SIMS experiments in a scanning fashion in order to determine the localization of the different compounds in presence and form an image of the object. The same group used a similar analytical approach in order to study the structure of drug bead systems of different encapsulated in amylose or Eudragit L30-D [75].

However, proteomic analysis is currently emerging as a complementary analytical methodology capable of providing information regarding the impact of the contact of the biomaterial with the organism or after implantation. Thereby, proteomic analysis gives the opportunity to attribute subtle changes in protein expression levels consequently to the exposition to the biomaterial or to monitor the proteins involved in tissue regeneration. Thus Kuo et al. used systematic proteomic analysis in order to improve the mesenchymal stem cells (MSCs) expansion and differentiation in bone marrow and Wharton jelly. In order to identify the proteins involved in these biological process, bone marrow and Wharton jelly samples were analyzed using differential 2D-DIGE to determine the proteins exhibiting expression levels significantly different between bone marrow and Wharton jelly. In total, 32 proteins demonstrated significant differences in expression levels could be selected using this approach. The proteins were then further characterized by MALDI–MS/MS analysis to obtain their unambiguous identification. Finally, the expression of the identified was knockdown in order to study their influence of the tissue regeneration. Results showed that lowering the expression of transgelin-2 allowed to increase the growth rate of MSCs. Also, knockdown of HSP90β had the effect to increase bone nodule formation and stopped cell growth [76]. MS-based proteomic analysis was also extensively used to characterize the extracellular matrices (ECM) [77]. Therefore Naba et al. studied ECM proteins originating from pancreatic islets, during angiogenic switch and subsequent insulinoma development. In this study the pancreatic islets samples were first undergoing decellularization in order to fractionate the proteins

originating from ECM. The samples were then prepared by performing reduction/alkylation followed by tryptic digestion. The peptide mixtures generated from the sample preparation were then separated and analyzed by nanoLC−MS/MS analysis. Because the acquisition rate of typical high resolution MS instrument is important enough, the nanoLC−MS/MS experiments allowed to provide a sensitive analysis of this sample composed of a mixture of several thousands of peptides. MS/MS data interpretation enabled the confident identification of the protein composing the ECM samples. Thereby, 120 ECM proteins could be successfully identified using nanoLC−MS/MS data. Consequently to the identification the pancreatic ECM proteins, the MS signal intensity corresponding to the specific *m/z* ratio of their peptides was monitored throughout their development (before/after angiogenic switch and as insulinomas). The results exposed show that 35 proteins were exhibiting significantly different expression levels during the development. This study clearly demonstrates the possibility to use MS-based proteomic analysis to monitor the expression levels of proteins in different conditions. Also, the authors suggest the expression levels of the proteins highlighted from the nanoLC−MS/MS proteomic analysis may be influenced to improve tissue regeneration in the context of diabetes for instance [78]. In a recent research article, Garcia-Puig et al. implemented a similar analytical workflow to investigate the protein content and expression levels of zebrafish ECM during heart regeneration. To enable the proteomic analysis of ECM, the heart samples were first undergoing decellularization with the aim of preventing contamination by protein originating from the intracellular medium. Consequently to this preparation, the samples were analyzed by nanoLC−MS/MS using a high-resolution MS instrument. MS/MS data were used to identify unambiguously the proteins composing the sample, and the intensity of the MS signal was considered to estimate the expression levels on the different proteins. The nanoLC−MS/MS experiments performed on control samples containing both ECM and intracellular medium, enable to successfully identify 447 different proteins. After sample decellularization, 36 proteins strictly belonging to ECM were identified. By considering the MS signal, they could attribute during tissue regeneration, the increase of the expression levels of several types fibrinogen, fibronectin 1b and periostin b. At the same time, collagen and fibrillin 2b levels were significantly lowered [79].

Also, MS/MS spectra providing the fragmentation of the identified peptides can enable the specific identification of peptide backbone PTMs which are involved in biological regulation and signaling [80−82]. Therefore proteomic experiments can be implemented in conjunction with complementary analytical approaches such as genomics and FC in order to obtain a detailed understanding regarding the implication underlying the implantation of biomaterials [83]. The information provided by proteomic experiments can provide decisive information regarding implantation outcome which can help to tailor the development of innovative biomaterials or reject early materials with potential to generate important adverse effects [84]. Note, conventional MS analysis was previously used to investigate the nature of the protein naturally adsorbing onto the surface of biomaterials upon exposure.

Thus Castner et al. have developed a method using TOF-SIMS analysis in order to identify adsorbed proteins after incubation of 2 hours in 1% bovine serum in the case of mica, polytetrafluoroethylene (PTFE) and silicon wafer. Results obtained have demonstrated the identification of several types of proteins, including serum albumin, fibronectin, fibrinogens, immunoglobulin G, and γ-globulins. As expected, they correspond to the most abundant proteins in the serums; nevertheless, the article shows the possibility using this analytical technique to identify unambiguously the adsorbed proteins [85,86]. Still, the literature regarding natural protein adsorption on biomaterials is scarce most likely due to the frequent introduction of novel media. However, the characteristics and performances concerning sensitivity and specificity of state-of-the-art proteomic analysis open up a new pathway for the comprehensive identification of proteins adsorbed to the biomaterial surface. In parallel, because recent research articles describe the development of biomaterials incorporating proteins into their structures, like growth factors or cytokines able to modulate the immune response, for their release after implantation [9]. In this case as well, recent researches show that the implementation of proteomic analysis is particularly interesting to monitor the release of the incorporated proteins. Indeed, because the identification of the protein is strictly based on high-resolution MS analysis of the generated proteolytic peptides, the identification is extremely specific. Therefore the proteomic analytical workflow is capable of identifying without ambiguity a single protein in a mixture containing several hundreds. As an illustration, Barthes et al. developed a biomaterial composed of gelatin cross-linked using transglutaminase which can be used to mimic ECM and enable cell proliferation. In order to enable cell development even in the absence of serum, the medium was loaded with several serum components such as albumin, serotransferrin in addition to different growth factors like epidermal growth factor (EGF) and fibroblast growth factor (FGF). To investigate the gradual release of the proteins incorporated to the media, gel samples were incubated in phosphate buffered saline (PBS) solution. The supernatants were then collected and submitted to conventional proteomic sample preparation—reduction/alkylation followed by overnight tryptic digestion. The peptide mixtures obtained from the sample preparation were then characterized by bottom-up proteomic analysis. Thus the interpretation of the MS/MS spectra allowed the identification of the proteins initially incorporated to the gelatin-based media. Also the results achieved from the proteomic analysis suggest their rapid release from the medium [87].

The proteomic analytical methodology based on high-resolution MS appears as a major technique to study the nature and expression levels or proteins composing biological samples. As a consequence, the large amount of information obtained from this type of analysis appears particularly attracting to improve the knowledge regarding biomaterials impact. Because proteomic is a demanding analysis from an experimental point of view which needs extensive optimization especially in regard to the matrix, further improvements can be expected in the upcoming future to improve the suitability between samples obtained from biomaterial implantation and the proteomic characterization.

4.4 Analytical methodologies adapted to protein structural characterization

Consequently to their introduction, the first generation of biomaterials was designed and produced with the conceptualization to represent inert supports which could sustain prolonged contact with organic tissues while having minimum biological implications on the host organism. In the recent years the research concerning the development of innovative biomaterials has demonstrated to be particularly prolific which progressively led to a shifting of that definition. As the knowledge regarding biomaterials progresses, they nowadays tend to represent complex and organized media which may incorporate into their structure various components, including proteins, implemented to be released in the direct surrounding environment. The incorporation of proteins to the structure of biomaterials is primarily driven to influence the interaction between the biomaterial and tissues [18]. The addition of proteins to biomaterials gives the opportunity for further functionalization. As an illustration, the integration of different types of cytokine appears as an elegant means to modulate the immune response after implantation, in order to limit inflammation or other adverse effects [88]. In some applications the proteins can be used on the contrary to stimulate the immune response in a localized region [89]. Different types of proteins have been successfully integrated to the structure of biomaterials, including growth factor such as vascular endothelial growth factor [90] and bone morphogenetic protein 2 [91], heparin [92], mAb [93], and granulocyte−macrophage colony-stimulating factor [94].

In the context of additional functionalization of biomaterials by protein integration, the prolonged contact between the protein and the biomaterial may influence in a dramatic manner the structure of the protein. Modification of the structure of the protein can result in various effects such as lowered or inhibited biological activity to, in the worst case, triggering of the immune response [95,96]. Thereby, it is crucial to assess the structural integrity of the protein upon contact with the biomaterial to ensure an unaltered biological activity. The stability of proteins can be compromised through a variety of processes. For instance, protein aggregation is attributed to partial or complete denaturation of the protein, exposing large part of the peptide backbone to the environment. Aggregation is then observed due to electrostatic and hydrophobic interactions, which primarily induces premature clearing [97]. Therefore protein aggregation due to the incorporation inside the structure of the biomaterial could result in elimination and subsequent altered biological activity. In the case of biopharmaceutical products for example, aggregation has been described as a critical quality attribute (CQA) by the regulation agencies like the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA) Therefore, the approval of this category of therapeutics is conditioned by a detailed characterization concerning that aspect is mandatory [98]. Proteins incorporated to biomaterials may also undergo PTMs. PTMs describe chemical modifications of amino acid residues which may be endogenous like environmental conditions or triggered from the organism to induce signaling [99−101]. Proteins

may exhibit a wide variety of PTMs such as methionine oxidation or asparagine deamidation (Fig. 4.8), principally depending on the type and localization of amino acid residue on the peptide backbone [102]. In the case of incorporation to the structure of biomaterials, the occurrence of PTMs may influence significantly the biological activity of the released protein potentially exhibiting altered efficacy or rapid elimination [103]. As an illustration, for biopharmaceutical products currently approved for therapeutic use such as mAbs, regulation agencies have positioned as CQA a complete set of PTMs hotspots which differs depending on the nature of the mAbs. Therefore these PTMs require mandatory characterization due to their known impact on the activity and pharmacological activity of the mAb, in order to envisage approval [104–106].

As a consequence, there is an increasing need for structural characterization of proteins incorporated to biomaterials to demonstrate in a comprehensive way the biological functionality upon release from the media to the direct environment of implants. In addition, investigations regarding the conservation of the structure of proteins released from biomaterials can provide complementary data supporting the beneficial effect of this type of approach. Protein represents highly complex macromolecules potentially with a wide range of micro heterogeneities. Due to their inherent complexity, the structural characterization of proteins requires dedicated analytical methodologies. Thus some major improvements both from technical

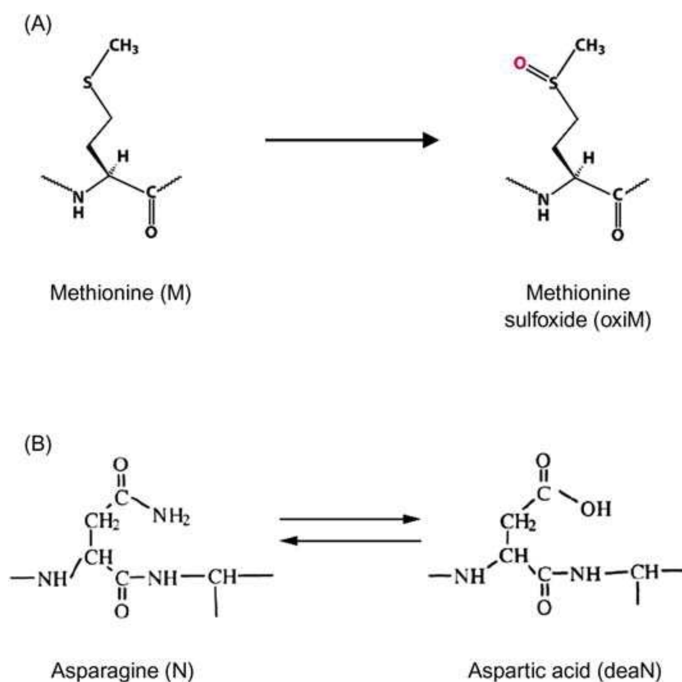

Figure 4.8 Schematic representation of posttranslational modifications (A) methionine oxidation and (B) asparagine deamidation.

developments and methodological strategies have been recently described in the literature in order to address the complexity of the structural characterization of biological macromolecule-like peptides, proteins, or carbohydrates [107,108]. Consequently to the introduction of ionization techniques enabling the analysis of biological macromolecules [27], MS has rapidly demonstrated its ability to play a pivotal role in the characterization of proteins over the different levels composing their structure [30]. Thereby, MS can be used to analyze the primary structure of proteins [109] as well as secondary [110] and higher order structure of proteins [111]. This is explained by the powerful specificity delivered by contemporary MS instrumentation as well as relevant sensitivity, in addition to the possibility to obtain detailed structural information using dedicated workflows.

The PTMs potentially affecting proteins are concerning specific amino acid residues leading to their respective outcome. The analysis requires then to focus on the primary structure of proteins in order to finely localize the position and specifically monitor the occurrence of PTMs over the entire peptide backbone. Therefore the characterization of PTMs is performed by the intermediate of a peptide-centric MS/MS analysis. The analytical workflow used for the characterization of PTMs is mainly derived from bottom-up proteomics analysis with partially differing constraints and objectives. Prior to the analysis, the sample containing the protein of interest is undergoing digestion using proteolytic enzymes in order to generate a mixture of peptides. Afterward, the peptide mixture obtained is separated and characterized by different types of MS/MS based experiments.

4.4.1 Sample preparation

Similarly to bottom-up proteomic experiments, sample preparation represents a key step to achieve a highly detailed characterization of the primary structure of proteins. This is primarily achieved by an optimal digestion of the peptide backbone. Therefore prior to the enzymatic digestion, protein disulfide bridges are reduced, conventionally using DTT and the reactivity of free thiols is inhibited through alkylation mainly using IAM. The use of these chemical reagents proved through numerous research article the possibility to perform the reduction–alkylation process of disulfide bridge in a rapid and robust manner. Afterward the protein is subjected to proteolytic digestion which is commonly performed using trypsin. However, unlike bottom-up proteomic experiments, the structural characterization requires to achieve complete sequence coverage of the peptide backbone in order to have access to the different sites potentially present on the protein. As a consequence, the use of different proteolytic enzymes such as serine proteases (GluC or LysC), metalloprotease (AspN), or chymotrypsin can be implemented due to their respective digestion specificity in order to generate peptide mixtures favorable with the subsequent MS/MS experiment and extended sequence coverage [43]. When the buffer conditions used for the digestion are similar, several proteolytic enzymes can be used in conjunction to induce further digestion of the protein backbone [112–114]. Alternatively, the sample can be submitted in parallel to different proteolytic digestion which MS/MS analysis results can be crossed in order to

maximize the coverage of the peptide backbone. Nevertheless, in the context of structural characterization, sample preparation should be thoroughly designed to maximize coverage depending on the respective amino acid sequence of the studied protein. In addition, because the sample preparation required is particularly extensive, it is of utter importance to optimize the reaction conditions in order to limit as much as possible the occurrence of PTMs originating from the sample preparation. Indeed as an illustration, the alkaline conditions used to commonly perform tryptic digestion are described to induce endogenous deamidation of the protein [115]. Still, careful optimization of the sample preparation protocol allows to limit that effect [116]. Also, the inclusion of a control enables to easily alleviate the production of endogenous modifications due to the sample preparation.

4.4.2 Tandem mass spectrometry−based analysis of posttranslational modifications

The protein mixture generated from proteolytic digestion sample preparation is afterward separated and characterized using MS. The analysis is conventionally performed using nanoLC−MS/MS instrumentation implementing C18 reverse stationary phase which porosity has been optimized for the separation of peptides. In the case of sample available in readily quantities, the analysis can be performed using ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) instrumentation. Due to the nature of the stationary phase and the direct hyphenation with the mass spectrometer, the mobile phase used to perform the LC is generally composed of a mixture of water and acetonitrile containing 0.1% FA [117]. Compared to shotgun proteomic experiments described previously, the initial protein complexity regarding the number of proteins is expected to be reduced when performing structural characterization which explains that short-time gradient can be implemented without compromising on the MS/MS characterization efficiency. However, the separation conditions should be carefully optimized in order to enable estimation in a reliable manner of the level of modification for each PTMs site. Thereby, the chromatographic separation should enable a systematic separation of the unmodified peptide from its counterpart experiencing the modification of one amino acid residue. That condition allows to prevent misinterpretation due to coelutions in addition to cancel the occurrence of competition effects during the ESI which could result in biased estimation of the levels of modification [118].

More recently, capillary zone electrophoresis coupled with MS/MS (CZE−MS/MS) was proposed as an alternative for the characterization of the primary structure of proteins, including highly comprehensive analysis of PTMs [119]. Indeed, the separation mechanism provided by the electrokinetic separation demonstrated to be particularly suitable for this type of analysis, with the possibility to achieve systematically a complete sequence coverage from tryptic digests in a reproducible manner. This characteristic is attributed to the ability of the electrophoretic separation to separate and transfer peptides to the MS instrument in a robust way regardless of

their chemical composition [109]. The selectivity delivered by CZE demonstrated the ability to systematically separate peptides exhibiting different types of PTMs from their intact homologues therefore enabling a site-specific and confident evaluation of the level of the protein modification. Thereby, that characteristic could be demonstrated in the case of N-terminal glutamic acid cyclization (pyroGlu), asparagine deamidation (deaN), methionine oxidation (oxiMet), and aspartic acid isomerization (isoD) (Fig. 4.9) which are described as naturally occurring PTMs [22,121].

For the later one, note that isomerization of aspartic acid does not imply a mass difference between the modified peptide and the corresponding intact one which without separation could then not be attributed based on the MS analysis alone. However, because CZE–MS/MS demonstrated the systematic separation of the peptides experiencing aspartic acid isomerization, the two respective forms could be successfully identified meaning the electrophoretic separation implemented in this particular case allowed to further enrich the level of characterization achieved regarding the analysis of this challenging PTM [120]. Finally, the separation of peptides exhibiting PTMs from their intact counterpart was maintained regardless of the global amino acid sequence of the peptide. The performance of CZE–MS/MS analysis appears particularly interesting for the characterization of PTMs because, in order to provide an optimal estimation of the levels of modification, separation of the modified PTMs is mandatory [122].

Consequently to their chromatographic or electrophoretic separation, peptides are gradually transferred to the MS instrument. The instrument performs the

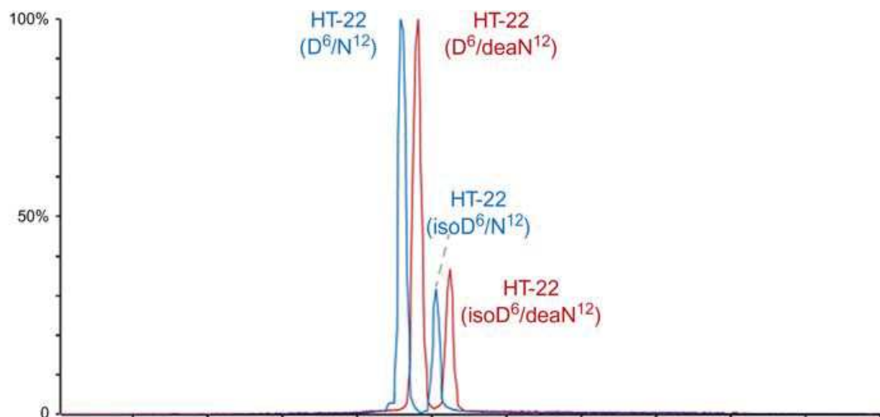

Figure 4.9 Extracted ion electropherograms of *m/z* values corresponding to HT-22 synthetic tryptic peptide (seq. FNWYV**D**GVEVH**N**AK) showing separation of (peak 1) unmodified HT-22, (peak 2) HT-22 exhibiting an asparagine deamidation in position 12 (D^6/deaN^{12}), (peak 3) HT-22 with isomerization of the aspartic acid residue in position 6 (isoD^6/N^{12}), and (peak 4) HT-22 affected by both PTMs (isoD^6/deaN^{12}).
Source: Adapted with permission from [120]. ©2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

measurement of the *m/z* ratio of the compounds eluting from the separation system (MS analysis) which is directly followed by the selection and fragmentation of the parent ion corresponding to the detected peptide.

The *m/z* ratios of the fragment ions are then measured (MS/MS analysis). MS/MS analysis is required in order to precisely position PTMs on the peptide backbone. To induce the fragmentation, CID is principally used. This is due to its wide availability in MS instrumentation in addition to the robust and relevant fragmentation obtained in the case of peptides using CID. However, electron transfer dissociation, which in particular has the capacity to reach higher fragmentation energies than CID, demonstrated to be interesting for the characterization of PTMs as well [123]. The identification of the peptide is achieved on the concomitant correlation between the high-resolution MS measurement of the peptide provided by MS analysis in addition to the fragmentation spectra obtained from MS/MS analysis (Fig. 4.7). Thus because CID fragmentation yields mainly b- and y-ions (Fig. 4.7B), therefore, the amino acid sequencing of the peptides can be precisely characterized by tracing the both series of ions (Fig. 4.10A). In most cases the occurrence of PTMs is involving a mass shift compared to the equivalent peptide without modification like C-terminal glutamic acid cyclization (−17.0265 Da), methionine

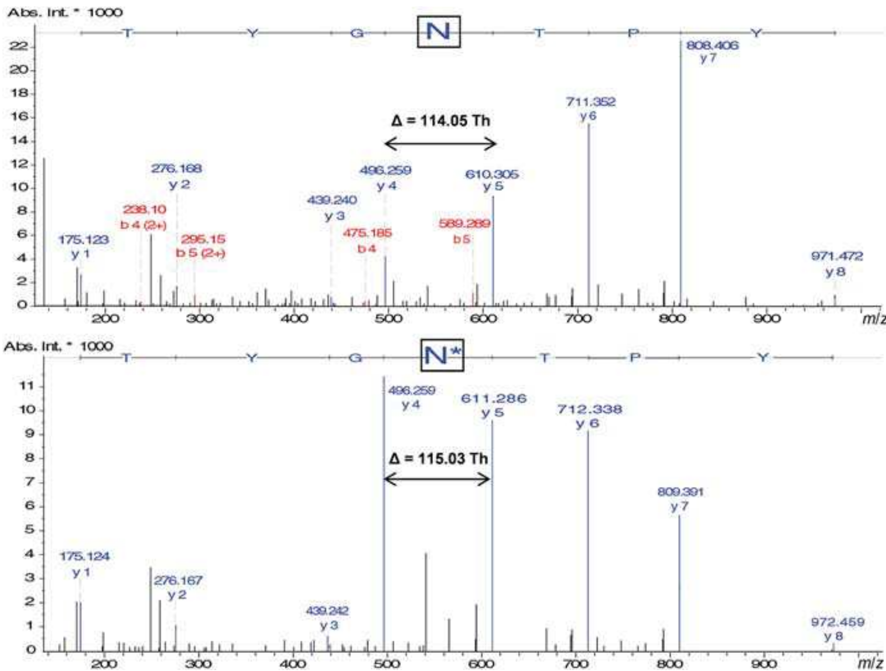

Figure 4.10 Example of MS/MS spectra of a tryptic peptide (IYPTNGYTR) showing the identification (A) without modification and (B) with a deamidation on Asn^{55} (N*) illustrating the partial modification of the protein. *MS/MS*, Tandem mass spectrometry.
Source: Adapted with permission from [109]. ©2013 Taylor and Francis publishing.

oxidation (+15.9995 Da), or asparagine deamidation (+0.9840 Da). Because CID provides a gradual fragmentation of the peptide backbone, the mass increment corresponding to a modified amino acid residue present will be equal to the mass of the concerned amino acid plus/minus the mass shift of the PTMs as emphasized in Fig. 4.10. Therefore the interpretation of MS/MS spectra provides unambiguous information regarding the nature of the PTMs alongside to the precise attribution of the amino acid residue affected even if several amino acids of the same type are present on the peptide. Complementarily, the identification of the peptide allows to localize precisely the modification on the peptide backbone of the protein which enables to correlate its impact on the biological activity of the protein. Sequence coverage is therefore primordial in that aspect in order to map in an extensive manner the occurrence of PTMs over the entirety of the peptide backbone of the protein. In particular, sample preparation and analytical conditions, including MS parameters, require a complete optimization to enable a maximized detection of the peptides composing the mixture. For each PTMs site identified through MS/MS spectra interpretation, the level of modification of the protein is estimated by comparing the intensity of the signal on the MS analysis between the modified peptide and the intact homologous. For that matter, it is important that both peptides are eluting at different times prior to their introduction to the MS instrument in order to prevent any biases in the estimation.

Due to the robustness of this analytical approach, supported by high resolution MS analysis, the characterization of PTMs has been extensively used to map the occurrence of PTMs on proteins in vivo [124]. This methodology is also at the moment extensively used for the characterization of biopharmaceutical products to monitor PTMs described to have an adverse influence on the activity/functionality of proteins used for therapeutic purpose [125]. Therefore it is possible to envisage the study of the occurrence of PTMs on proteins which may be generated by their incorporation to a biomaterial. Indeed, the MS/MS spectra recorded from the analysis of the protein collected from the release of the biomaterial can be used to localize eventually the presence of PTMs on the peptide backbone. In parallel a similar analysis realized as a control experiment on a sample of the protein which was not incorporated to the biomaterial could further demonstrate the origin of the PTMs (Fig. 4.10). Considering the performance of the current instrumentation, this type of characterization is particularly simple to implement and should be developed in the near future, especially in the latter stage of innovative biomaterial development.

4.5 Conclusion

The research regarding innovative biomaterials especially targeting tissue regeneration involves a synergy of disciplines and expertise. The primary purpose of biomaterials is involving long-term interaction with highly complex biological matrices. In the meantime, major breakthroughs have been achieved in biology to develop advanced analytical methodologies able to provide extensive information regarding

biological systems and their evolution over time. Proteomics is a prominent example of this category of analytical strategies as it provides the protein content of complex biological samples in unprecedented details with the possibility to monitor their respective expression level. Therefore it even allows to access information regarding biological signaling which cannot be obtained from genetic analysis delivering a complementary insight regarding the studied organism. The conception of innovative biomaterials can profit from the implementation of this type of advanced methodologies, once strictly reserved to fundamental biology investigation. In conjunction with the integration of other types of analytical strategies such as genomics or FC, the use of those tools can help to understand the complex biological implications of the integration of biomaterials for tissue regeneration. Thus because of their technical maturity, such techniques can be employed in various context. For instance, proteomic analysis can be implemented to scale subtle changes in the protein profile upon biomaterial exposure or implantation in order to interpret biological reactions. The analytical information provided can help to tailor biomaterials with improved compatibility with the biological environment. MS tends to play a key role in the comprehensive characterization of protein structure. This technique can also be used to deliver information regarding the structural integrity of protein that has been incorporated to biomaterials. That aspect appears particularly important to ensure maintained activity of proteins integrated to the structure of biomaterials which is a crucial requirement to retain the functionalization sought for the biomaterial. In this case as well, dedicated characterization can help to point endogenous structural instability originating from exposition to the biomaterial in order to design compatible media. With the constant improvement of MS-based analytical methodologies such as the introduction of glycomics and lipidomics, knowledge regarding biomaterial outcome are expected to be further improved in the future.

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