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The application of MALDI TOF MS in biopharmaceutical research

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ABSTRACT

The development and quality assessment of modern biopharmaceuticals, particularly protein and peptide drugs, requires an array of analytical techniques to assess the integrity of the bioactive molecule during formulation and administration. Mass spectrometry is one of these methods and is particularly suitable for determining chemical modifications of protein and peptide drugs. The emphasis of this review is the identification of covalent interactions between protein and peptide bioactives with polymeric pharmaceutical formulations using mass spectrometry with the main focus on matrix-assisted laser desorption/ionization (MALDI) coupled tandem time-of-flight (TOF/TOF) mass spectrometry (MS). The basics of MALDI TOF MS and collision-induced dissociation (CID)-based ion fragmentation will be explained and applications for qualitative characterization of protein and peptide drugs and their interactions with pharmaceutical polymers will be discussed using three case studies.

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1. Introduction

Biopharmaceuticals, and particularly protein and peptide drugs, are becoming increasingly more important as therapeutic agents in an era of modern biotechnology and targeted therapy (Delie and Blanco-Prieto, 2005; Mustata and Dinh, 2006; Oliva et al., 2007). Compared to small molecular weight drugs, protein and peptide drugs have more complex and specific three-dimensional structures, and are thus more challenging to formulate (Allemann et al., 1998; Oliva et al., 2007). Stability during formulation, storage and after administration needs to be ensured by designing pharmaceutical formulations capable of protecting the bioactive and maintaining their biological activity without affecting their chemical and physical integrity (Alonso, 2004; Bilati et al., 2005). The Food and Drug Administration (FDA) defines a drug as stable when no more than 10% deterioration occurs over a period of at least 2 years (Bilati et al., 2005). The definition of stability for biopharmaceutical drugs, however, needs to be more accurate and comprehensive as a result of their more complex chemical structure. In this article we differentiate chemical stability, which refers to the integrity of the primary structure (the amino acid sequence) and physical stability, which refers to the secondary and tertiary structure, i.e., the folding of the macromolecule. Several processing and formulation parameters can affect the chemical and/or physical stability of proteins and peptides, which may compromise bioactivity. Chemical modifications of the amino acid sequence may involve deamidation, isomerization, racematization, oxidation, disulfide formation, β-elimination or hydrolysis of peptide bonds (Niu, 1998), but also covalent interactions of the biopharmaceutical with the pharmaceutical carrier (Gibaud et al., 1998; Kafka et al., 2009; Tasset et al., 1995). As a consequence, the adoption of the very specific three-dimensional conformation of the macromolecule required for biological activity may be compromised, potentially altering receptor binding affinities, antigenicity or sensitivity to *in vivo* proteases (Bummer and Koppenol, 2000).

The complexity of protein and peptide biopharmaceuticals requires use of a wide range of analytical methods to characterize and analyze them to assure their stability before and after formulation (Hoffmann, 2000). Contemporary analysis in the development and quality assessment of protein and peptide drugs utilizes predominantly high-performance liquid chromatography (HPLC) as a separation method in combination with a range of different in-line detectors such as ultraviolet (UV), fluorescence, evaporative light-scattering (ELS), refractive index (RI), chemiluminescent nitrogen (CLN) and charged aerosol (CA) detection (Issaq et al., 2009; Hoffmann, 2000). Major disadvantages of these detectors include the lack of compound specificity, the requirement for analyte modification prior to analysis or non-linear response of the detector to analyte concentration (Baertschi, 2006; Issaq et al., 2009).

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Spectroscopic methods may also be used for the identification and characterization of compounds, such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) (Baertschi, 2006). They can both be coupled either in-line or off-line with separation methods, such as HPLC, and are invaluable as complementary, orthogonal methods in the characterization of biopharmaceuticals and for impurity profiling. Complementary methods are needed for characterization beyond the first impurity screening with one-dimensional methods (e.g., UV-HPLC) and for enhanced profiling.

NMR is a very powerful tool for the characterization and identification of molecular species, revealing structural information of the analytes on the atomic level. However, the high costs involved, and the high purity of samples and technical expertise required, leave NMR spectroscopy less utilized than it probably should be. MS, on the other hand, is a more affordable and universal method of detection, providing valuable information on the identity of analytes on the molecular level (Baertschi, 2006; Vuletic et al., 2005). MS detection has diverse applications whenever accurate mass measurements with high sensitivity are required (Hernandez et al., 2008; Holcapek et al., 2008; Le Bizec et al., 2009; Maurer, 2007). The high throughput, high sensitivity and selectivity required for the analysis are major advantages of MS. As a second separation dimension, liquid chromatography (LC), in-line coupled to MS can simultaneously detect different molecular species that co-elute at the same time and cannot be discriminated by HPLC alone (Ho et al., 2008; Issaq et al., 2009; Vuletic et al., 2005). Mass spectrometry is the method of choice for the detection of any changes in the primary structure of a bioactive compound (Hoffmann, 2000; Koh et al., 2003). Changes in secondary and tertiary structure can be detected with mainly physical, spectroscopic methods (Hoffmann, 2000), such as circular dichroism (CD), Fourier transform infrared (FT-IR) spectroscopy (Haris and Severcan, 1999; Hoffmann, 2000; Jørgensen et al., 2004), calorimetric techniques, such as differential scanning calorimetry (DSC) (Bummer and Koppenol, 2000; Li et al., 2007) and fluorescence spectroscopy, which are not discussed further in this review.

Polymers are frequently used in the biopharmaceutical field in order to design oral delivery systems that can protect the bioactive against pH challenges and enzymatic degradation, facilitate uptake of the bioactive, target specific tissues, and release the drug in a controlled/sustained way whilst maintaining the bioactivity of the drug (Couvreur et al., 2002; Soppimath et al., 2001; Vauthier and Bouchemal, 2009; Vauthier et al., 2003). In addition to common instabilities, such as oxidation or aggregation of proteins and peptides, the chemical modification of macromolecules due to covalent interaction with the drug carrier is an important issue during formulation and storage. Knowledge of the specific site of modification, and thus enhanced insight into possible interaction mechanisms, provides invaluable information for the formulator. The main focus of this review is the characterization of chemical modifications in protein and peptide biopharmaceuticals occurring in polymeric delivery systems due to the covalent conjugation of the bioactive to the polymer using a recent mass spectrometric method: matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) coupled with collision-induced dissociation (CID) tandem mass spectrometry (MS/MS). The strength of this method is not only the ability to detect traces of minimally modified bioactives in the presence of unmodified compound, but also the ability to identify the site of modification within the bioactive molecule. In the following paragraphs, MS will be introduced with a focus on MALDI TOF MS as an analytical method and contemporary applications for the analysis of isolated proteins and polymers will be discussed. In the second part of the review we elaborate the recent establishment of MALDI TOF MS for the characterization of peptide–polymer conjugates using three case studies.

2. Mass spectrometry instrumentation

In the following section we will briefly describe the basics of ionization methods focused on electrospray ionization (ESI) and MALDI and will then discuss different mass analyzers suitable for the analysis of biomolecules and polymers used in biopharmaceutical research. The principles of mass measurement and ion fragmentation will be demonstrated using a tandem time-of-flight (TOF/TOF) mass spectrometer as an example. We refer the reader to excellent review articles (Domon and Aebersold, 2006; Yates et al., 2009) and text books (James, 2001) for further information about the principles of biological MS.

2.1. Ionization

Mass spectrometry separates ionized molecules based on their mass- and charge-dependent trajectories in electric and magnetic fields or measures their velocities upon ion acceleration in electric fields. Consequently, all molecular species to be analyzed by MS must be ionized prior to measurement. Many different techniques for the ionization of molecules prior to mass analysis have been developed. However, not all methods are suitable for the ionization of larger biomolecules, such as peptides and proteins. A significant milestone in the improvement of mass spectrometry techniques was the development of the two soft ionization methods ESI (electrospray ionization) (Fenn et al., 1989) and MALDI (Hillenkamp et al., 1991; Karas et al., 1987), which enabled new strategies to analyze large biomolecules and polymers (El-Aneed et al., 2009). In comparison to other ionization methods such as electron ionization, chemical ionization or fast atom bombardment, ESI and MALDI are mostly non-destructive to larger biomolecules, do not require chemical modifications and for most analyte molecules, result in high ionization efficiencies. Both ionization methods can be coupled interchangeably to various types of mass analyzers.

2.1.1. Electrospray ionization (ESI)

In ESI an acidified solution of analyte molecules (usually in a methanol/water or acetonitrile/water solvent) is sprayed through a fine capillary or emitter tip into a high voltage electric field (2–5 kV), which is applied between the liquid phase and the inlet of the mass spectrometer. The high charge density of the acidified liquid phase entering the high voltage electric field causes dispersion and formation of fine analyte/solvent droplets. Analyte molecules become ionized when they are transferred to the gaseous phase after desolvation of the solvent/analyte droplets. The fundamentals of ESI are extensively reviewed by Cech and Enke (2001). ESI is a very efficient and soft ionization method that mostly generates analyte ions with multiple charges. However, it is also very susceptible to interference with contaminants such as salts and other charged, small molecular weight compounds. Since the development of ESI, major improvements have been implemented, allowing the in-line coupling of liquid chromatography to the ESI process and the reduction of flow rates down to a few nanoliters per minute (nanospray ionization – NSI) to gain sensitivity. The in-line coupling of nanoflow liquid chromatography to NSI-mass spectrometry is one of the most sensitive high throughput mass spectrometry applications and became a routine analytical tool in proteomics and other disciplines.

2.1.2. Matrix-assisted laser desorption/ionization (MALDI)

In MALDI, the ionization of analyte molecules is assisted by a compound, the matrix, that can be activated by the absorption of laser energy (Montaudo et al., 2006). Sample and matrix are co-crystallized onto a solid phase target, usually a metal plate. Using a pulsed laser directed onto the sample/matrix crys-

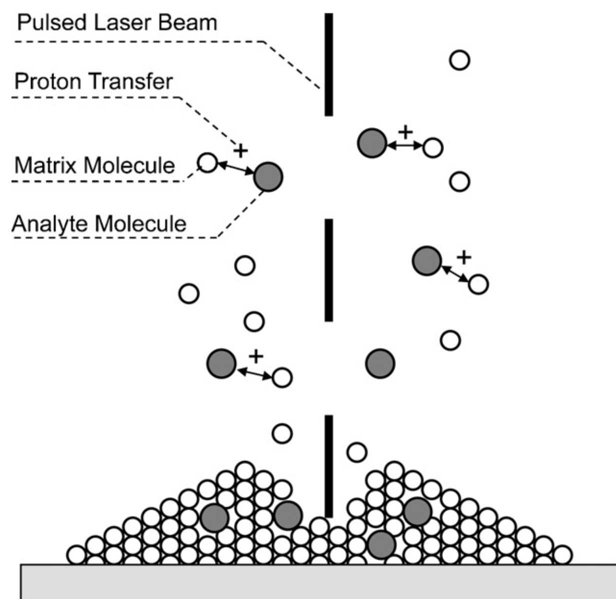


Fig. 1. Matrix-assisted laser desorption/ionization. Pulsed UV-laser energy is used to transfer matrix and analyte molecules from the solid phase support into the gaseous phase. In the transition from solid phase into the gas phase a charge transfer, usually a proton transfer, occurs between activated matrix and analyte molecules. The matrix can be a proton donor or acceptor, resulting in positively or negatively charged analyte molecules. The resulting charge state depends strongly on the nature of the analyte.

tals, activated matrix and analyte molecules are vaporized and released into the vacuum of the ion source (Montaudo et al., 2006). Desorption of molecules from the solid phase target and transition into the gaseous phase is followed by ionization, usually a proton transfer between analyte and matrix molecules (Fig. 1). The activated matrix can either be the proton donor or proton acceptor, generating positively or negatively charged analyte ions.

For smaller molecules, such as peptides and small proteins, MALDI generates predominantly singly charged ions ($z = 1$), which minimizes spectral complexity and simplifies the interpretation of mass-to-charge values (El-Aneed et al., 2009). Although the solid to gas phase transition and ionization of MALDI cannot be coupled in-line to liquid chromatography, interfaces have been designed that allow an efficient off-line coupling of liquid chromatography. For example, liquid-handling robotic interfaces are used to pre-mix eluting fractions with matrix and automatically spot them onto a metal plate. Such setups enable high throughput LC-based MALDI-MS experiments (Pan et al., 2009).

2.1.2.1. The matrix. Both choice of matrix and sample preparation have been shown to influence the nature of molecules that can be detected using MALDI TOF and the quality of the resulting data (Lastovickova et al., 2009; Nielen, 1999; Zhang et al., 2010). Excellent reviews are available on the different matrices used both in the analysis of isolated proteins and peptides and polymers (He and Chen, 2007; Montaudo et al., 2006; Nielen, 1999; Peacock and McEwen, 2006; Weidner and Trimpin, 2008). The appropriate choice of matrix, however, is highly dependent on the nature of the sample and involves optimization of the experimental conditions. MALDI TOF matrices should (i) be able to embed and isolate analytes (e.g., by co-crystallization); (ii) be soluble in analyte-compatible solvents; (iii) be vacuum-stable; (iv) absorb the laser wavelength; (v) initiate co-desorption of analyte upon laser irradiation; and (vi) promote analyte ionization (Chapman, 1996). Alpha-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), sinapinic acid (SA) and ferulic acid (FA) are the most common matrices used for the detection of proteins and peptides (Fenselau and Demirev, 2001; Zhang et al., 2010). The reasons they are the most commonly used matrices are that FA has been shown to be suitable for high-molecular-weight proteins (>15 kDa) (Fenselau and Demirev, 2001), and using CHCA, great sensitivity can be achieved at trace concentrations of a range of different proteins and peptides (fmol μl^{-1}) (Zhang et al., 2010). High-quality mass spectra were obtained for different glycoproteins using binary mixtures of matrices, such as mixtures of DHB and CHCA (Lastovickova et al., 2009). Matrices described in the literature for the analysis of synthetic polymers are very similar to the matrices found for protein analysis. The polarity of the matrix used for analysis of polymers should match the polarity of the polymer (Nielen, 1999) (Fig. 2).

Since the matrices used for the analysis of protein/peptide drugs and polymers are very similar, it is not surprising that the matrices found in the literature for the detection of protein/peptide–polymer conjugates comprise the same chemicals. Matrices commonly used for the detection of protein/peptide–polymer conjugates include for example CHCA (Kafka et al., 2010; Na et al., 2003) or SA (Boyer et al., 2007).

2.1.2.2. Sample preparation. In comparison to MALDI of proteins and peptides, the final ionization of polymers occurs via cationization rather than protonation (Nielen, 1999). Cations, such as silver or copper, may be added to the sample, but sodium or potassium adduct ions may be readily available due to their presence as impurities in glassware, solvents or reagents etc. (Nielen, 1999). The ionization of protein/peptide–polymer conjugates, however, may be performed without the further addition of cations, since the ions to be detected are protein/peptide-associated molecular species. The absence of unconjugated polymer in the mass spectra,

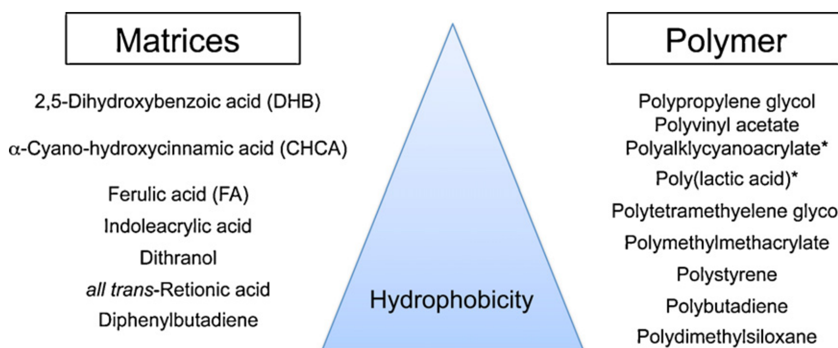


Fig. 2. Schematic representation using some examples to illustrate the relationship between hydrophobicity of polymer and matching matrix recommended for analysis (Nielen 1999); * polymers are discussed in more detail in this review (Section 4).

however, must not be misinterpreted as the absence of polymer in the sample. The fact that free polymers do not appear in the mass spectra, may simply be due to sample preparation conditions (i.e., no cationization salt added, only protonation through the matrix). For the preparation of the final mixture, ideally solvents are chosen that solubilize the matrix (and the cationization salt) and the sample in order to minimize the risk of sample segregation (Nielen, 1999). The molar ratio of sample to matrix is a critical parameter, determining whether a good mass spectrum will be obtained (Nielen, 1999).

To transfer the final mixture to the MALDI target, there are several methods that can be used. Generally, the sample can either be applied in one or two layers. In the one-layer technique (dried-droplet method), the mixture containing matrix (and salt) and analyte is spotted onto the target and air-dried. Fast drying techniques, such as drying under vacuum or under a high-purity nitrogen gas stream, have been used to speed the crystallization process and to minimize the risk of segregation (Nielen, 1999). Samples may also be prepared by the two-layer method (thin-layer method), where a layer of matrix is spotted first onto the target plate and allowed to crystallize, then the sample is added on top of the matrix and dried (Nielen, 1999). The application of the samples to the target by either method can be performed using a pipette or by electrospray deposition (Axelsson et al., 1997). An interesting modification of the conventional dried-droplet method was reported by Donegan et al. (2004). Pre-coating of the MALDI target plate with nitrocellulose and CHCA, resulted in a fractional crystallization of sample due to the increased hydrophobicity of the target. The dried sample spot showed an outer matrix-rich ring surrounding an inner smaller droplet containing primarily the sample. Significant matrix ion suppression was evident after desorption and ionization of molecular species in the inner droplet, which is particularly beneficial for the analysis of small molecules, since matrix ions usually interfere with samples in the lower mass region.

2.2. Mass analyzer

There is a great variety of mass spectrometers using various types of mass analyzers to separate and select ionized molecules, such as quadrupole-based analyzers and their derivatives, the ion trap (IT) instruments, and high resolution mass spectrometers with time-of-flight (TOF), Orbitrap or ion cyclotron resonance (ICR) mass analyzers.

Although quadrupoles are low resolution mass analyzers with only a moderate mass accuracy, the setup of three quadrupoles in series in a LC-coupled ESI triple quadrupole (ESI-QQQ) instrument facilitates a very fast and sensitive screening of known ion signatures in very complex matrices (Hüttenhain et al., 2009). These instruments are frequently used for the verification and validation of biomarkers and the detection of drugs and other small compounds in body fluids or other complex samples, and are therefore of great interest to biopharmaceutical research and industry. Many modern mass spectrometers are hybrid instruments designed to combine the strengths of different mass analyzers in one instrument. Usually, fast and sensitive IT instruments are coupled to very accurate ICR or Orbitrap analyzers, which combine fast and efficient ion fragmentation with extraordinary mass accuracy. The quadrupole mass filters and collision cells have been combined with TOF analyzers in Qq-TOF hybrid instruments, a very versatile group of mass spectrometer for various fields of application (Morris et al., 1996). TOF analyzers can measure molecules within a very broad mass range from small molecular weight compounds to large intact proteins and have therefore been widely used in various fields of research. The wide range of applications for non-hybrid TOF analyzers has been further extended with the development of a tandem TOF (TOF/TOF) mass spectrometer (Medzihradszky et al., 2000), which allows a specific ion selection and fragmentation to gain structural information for compounds of interest. Although these mass analyzers use different fundamental principles to separate and select ions, they all strictly measure the mass-to-charge ratio (m/z) of an ion.

2.2.1. Time-of-flight (TOF) separation of molecules

In TOF mass spectrometry, all ionized molecules are accelerated in an electrostatic field (usually 15–35 kV) towards a field free high vacuum tube region where different molecular species are separated by their m/z-dependent velocities.

Contemporary TOF analyzers achieve signal resolutions of up to 20,000 FWHM (full width at half maximum) resulting in peak widths of 0.1 Da for a singly charged molecule of 2000 Da. The mass accuracy is in the range of ca. 20–30 ppm with external calibration and 5 ppm with internal spectrum calibration. The latter is equivalent to a mass error of 0.01 Da for a 2000 Da molecule. Despite the high resolution and good mass accuracy, the intact mass of a molecular ion is often not sufficient for identification. In particular, the analysis of modified variants of compounds of interest requires detailed structural information to determine the nature and site of modification. In mass spectrometry, the fragmentation of ions in the gaseous phase inside the mass spectrometer and the analysis of specific fragmentation patterns are widely used to gain more detailed structural information (Box 1).

Box 1: The Time-of-Flight is proportional to the *m/z* ratio

Upon acceleration of ionized molecules in an electrostatic field, the potential energy zU (where z is the number of ionic charges and U the strength of the electric field) is converted into kinetic energy ½ mv^2 (where m is the mass of the molecule and v its velocity). When the electrostatic field is constant, the velocity of the accelerated molecule depends strictly on the mass (m) and number of ionic charges (z). When all molecules are singly charged, e.g., after MALDI, all ions gain the same kinetic energy and their velocities depend only on the mass, with the smallest mass gaining the highest velocity. The velocity of molecules is measured by the time they need to travel from the source region through the high vacuum tube to the detector. The time-of-flight (t^2) is proportional to the m/z ratio of the analyte Eq. (1), where k is an instrument-specific constant (Eq. (2)) considering the length of the flight tube and the voltage applied to accelerate the ions) and can be transformed into a m/z-based mass spectrum (Wollnik, 1993).

$$t^2 = \frac{m}{z}k \tag{1}$$

t = time, m = mass, z = charge number and k = constant.

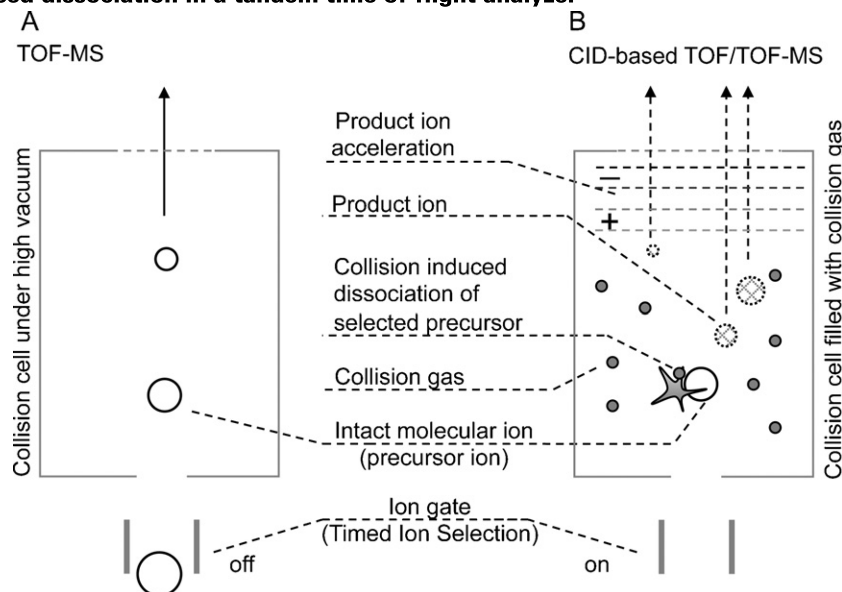
$$k = \frac{d^2}{2eU} \tag{2}$$

d = length of flight path and eU = potential energy.

2.2.2. Collision-induced dissociation (CID): fragmentation of molecules

In ESI- or MALDI-coupled mass spectrometry, the rate of spontaneous fragmentation of ionized molecules in the instrument is usually very low. Use of high laser fluencies, in MALDI for instance, can induce molecular fragmentation either in the ion-source region (referred to as in-source decay (ISD)) or in the field-free region of a TOF analyzer (referred to as post-source decay (PSD)) (Nielen, 1999). Although this method has been frequently used e.g., for peptide and protein sequencing with MALDI-TOF instruments, it lacks sufficient sensitivity due to inefficient fragmentation (Medzihradszky et al., 2000). More efficient fragmentation meth-

Box 2: Collision induced dissociation in a tandem time-of-flight analyzer



In a TOF/TOF analyzer two flight tubes are arranged in series and linked by an ion fragmentation device, the collision cell. The collision cell is equipped with an electrostatic ion gate for a specific ion (precursor or parent ion) selection and an additional acceleration grid for fragment ion (product or daughter ion) acceleration. (A) In MS mode, the collision cell is under high vacuum and the ion gate is switched off. All precursor ions can pass through to the detector recording a precursor mass spectrum. (B) When activated, the collision cell is filled with a neutral gas such as nitrogen or argon (collision gas) at a low pressure. The ion gate allows only precursor ions with a selected *m*/*z* value to pass through into the collision cell for CID. The resulting product ions are then accelerated and separated for a second TOF measurement. This setup allows the performance of the four consecutive steps required for a typical tandem mass spectrometry (MS/MS) experiment using a TOF/TOF instrument: (i) intact molecular mass measurement; (ii) specific precursor ion selection; (iii) fragmentation of selected precursor ion; and (iv) measurement of product ions.

ods have been developed suitable for different mass analyzers (Sleno and Volmer, 2004), such as high and low energy collision-induced dissociation (CID), electron capture dissociation (ECD) (Zubarev et al., 2000) and electron transfer dissociation (ETD) (Syka et al., 2004). CID is the most widely used method in the area of mass spectrometry of biomolecules, because it is compatible with the most frequently used mass analyzers, such as IT, QQQ, QqTOF and TOF/TOF.

Generally in CID, ions are accelerated or excited to a higher kinetic energy state and allowed to collide with atoms/molecules of the collision gas (Box 2). Every collision event increases the internal energy of the precursor ion, which eventually results in chemical bond cleavages (Wells and McLuckey, 2005). Peptide ester bonds, for example, are prone to cleavage under CID conditions. This can be used to sequence peptides based on the measurement of different product ions generated by the fragmentation of one precursor ion species (Biemann, 1990; Medzihradszky, 2005; Steen and Mann, 2004) or the detection of modified biomolecules, such as the discovery of peptide acylation products in degrading poly(lactic-*co*-glycolic aid) (PLGA) microparticles (Na et al., 2003). The fundamentals of peptide fragmentation pathways are extensively reviewed by Paizs and Suhai (2005).

2.2.3. Detection of molecules and interpretation of spectra

CID of a selected ion species (precursor ion) in the collision cell of a mass spectrometer generates a compound-specific fragment ion (product ions) pattern, which is highly reproducible under constant CID conditions. The product ion pattern together with the intact mass of the precursor ion can be used for compound identification. However, often CID-spectra are very complex and manual interpretation is difficult. Software tools are used to match the combination of measured precursor mass and product ion pattern with a known experimental pattern from large spectrum libraries. In the case of peptide sequencing/identification, large sequence databases are interrogated to find peptide mass and predicted fragmentation pattern that matches the measured spectrum. This strategy can also be used to identify large polypeptides and proteins (Palagi et al., 2006). Since CID is most efficient in the mass range of peptides, larger polypeptides and proteins are usually cleaved into smaller peptide units prior to the mass spectrometric analysis. An amino acid site-specific protease, such as trypsin, that cleaves C-terminally at Lys and Arg is used for a controlled enzymatic protein hydrolysis and the resulting peptides are analyzed by CID-MS/MS. In an automated data analysis procedure, the software predicts peptide masses by an *in silico* fragmentation of large protein sequence databases based on the sequence specificity of the proteolytic enzyme. The fragmentation of peptides is reproducible and can also be predicted based on the amino acid sequence. The measured intact peptide masses and CID product ion masses are then compared with predicted values. Identified peptide sequences are subsequently aligned with the protein sequences. Positive ion mode, low energy CID results in peptide bond cleavages, which predominantly yield two types of product ions, one carrying the N-terminus referred to as b-type ion and the other carrying the C-terminus referred to as y-type ion (Biemann, 1990). Further cleavage events can result in the loss of water (−18 Da), ammonium (−17 Da) or carbon monoxide (−28 Da) either from amino acid side chains or specifically from b- or y-type ions. The carbon monoxide loss from b-type ions, which is prevalent under conditions of high energy CID, results in an additional peak referred to as a-type ions. The a-ion occurs as a satellite peak of the corresponding b-ion and can be used as a diagnostic signal to confirm the b-ion series. Immonium ions are low mass product ions that are generated by multiple cleavage events. They represent a single amino acid side chain still bound to the alpha carbon with

the alpha amino group attached. Immonium ions indicate the presence of certain amino acids in the peptide but do not reveal their positions in the sequence. The sequence of amino acids is determined by a series of consecutive b or y-ions. Both ion series extend from the termini by increments according to the masses of following internal amino acids and are labelled with the type of ion and ascending subscript numbers with b_1 and y_1 being the N-terminal and C-terminal amino acids, respectively. An amino acid modification can be identified by a specific mass shift of one or a group of related ions in the CID MS/MS spectrum.

3. Limitations of MALDI TOF MS

Despite the broad range of applications for MALDI TOF MS in various areas of research, it also has its limitations, which should be discussed here. MALDI requires a chemical compound – the matrix – to be added in excess to the analyte for efficient ionization (as described above). Most matrices also form ions upon activation by UV-laser light. These matrix ions generate very strong signals in the low mass region (up to ca. m/z 500) of the spectrum. The high abundance of these matrix ions may result in a detector saturation, which suppresses signal intensities of ions with slightly higher m/z values. Usually a low mass ion gate is activated to deflect matrix ions and protect the detector. MALDI TOF MS is therefore not suitable for a reliable detection of low molecular weight compounds (below m/z 500).

Besides the low efficiency detection of low mass analyte ions in MALDI TOF MS, the detection of high molecular weight compounds also has its limitations. Although TOF analyzers have an outstanding mass range (up to 500 kDa is possible) they have an inherent lower sensitivity at the higher molecular weight region, which is due to lower impact velocity of high-molecular weight ions (Axelsson et al., 1996; Nielen, 1999). This problem occurred in the study of Boyer et al. (2007) and is mentioned in Section 4.2. In their study, high molecular-weight protein-polymer conjugates were produced and the detection limits of the mass analyzer exceeded with molecular weights of 100 kDa and higher. Complementary methods (SEC, NMR) were applied to confirm the results obtained by MALDI TOF MS.

The most important limitation of MALDI coupled mass spectrometry is suppression of ionization when a complex mixture of molecules is analyzed in a single sample. Molecular species with high ionization efficiencies dominate the mass spectrum whereas species with the same abundance but low ionization efficiencies are suppressed (Krause et al., 1999; Wenschuh et al., 1998). Consequently, the resulting mass spectrum may display only a subset of the sample. A sample purification or fractionation can be performed to separate the different types of compounds and reduce sample complexity. An HPLC-based pre-fractionation prior to MALDI MS is usually performed for very complex samples. The limitation of suppression of ionization, however, can also be advantageous for certain types of analyses. For example, if low abundance compounds with high ionization efficiency are analyzed in the presence of high abundance compounds with a lower ionization efficiency, the resulting mass spectrum will show good signal intensities of the low abundance compounds. Below we give an example of such an analysis (Section 4.1) where we have used this limitation of MALDI for our benefit to detect peptide–ECA co-polymers in the presence of high amounts of ECA polymer (Kafka et al., 2009).

4. Application of MALDI TOF MS to the analysis of biopharmaceuticals

MALDI TOF MS has become an important analytical tool in the field of pharmaceutical research. We will discuss how MALDI TOF MS has been used to qualitatively characterize covalent interactions of protein and peptide drugs with three different polymers commonly used in the pharmaceutical field. The three different case studies reviewed here, demonstrate the potential diversity of protein and peptide drugs to covalently interfere with polymers at various stages of formulation and also storage. The nature of the polymerization process and the time at which the bioactive is added to the formulation are two key factors determining the potential for covalent conjugation. *In situ* forming acrylic polymers (Section 4.1.1 and 4.1.2) are compared to pre-formed poly(lactic-*co*-glycolic acid) (Section 4.1.3) polymers. By comparing two different methods of *in situ* polymerization, it also becomes clear, how critical it is to be able to control the event of conjugation. Uncontrolled conjugation processes within the pharmaceutical formulation are unwanted leading to loss of utility for application in human medicine. In contrast, controlled conjugation of protein and peptide drugs with polymers via controlled free radical polymerization mechanisms has become important for applications in medicine, biotechnology and nanotechnology (Boyer et al., 2007; Duncan, 2003).

In order to analyze protein/peptide–polymer conjugates in biopharmaceutical formulations, the analysis conditions need to be optimized to match the nature of proteins/peptides as well as the nature of the polymers. Whilst the analysis of proteins and peptides using MALDI TOF MS is well established, the analysis of polymers is more complex due to the co-existence of several molecular weight distributions that can be present in a sample (Nielen, 1999). According to Nielen (1999) (i) synthetic polymers are polydisperse and thus have a molecular weight distribution (MWD) instead of a single molecular weight; (ii) polymers might exhibit different end group functionalities due to different initiation and termination processes (functionality type distribution, FTD); (iii) for copolymers, such as poly(lactic-*co*-glycolic acid), there is an additional chemical composition distribution (CCD) and (iv) an architecture distribution (linear, branched, dendritic, etc.); and (iv) block-copolymers also exhibit sequence and block-length distributions. Depending on the conditions under which protein/peptide–polymer conjugates are produced, it may be expected that the conjugated polymers, similar to the parent polymers, comprise polydisperse systems and thus exhibit a MWD, FTD etc.

4.1. In situ polymerization of poly(alkylcyanoacrylate) (PACA)

The first case study is the pharmaceutical application of the polymer poly(alkylcyanoacrylate) (PACA). This polymer has been extensively studied for more than 30 years and many studies have demonstrated the potential of this polymer for the delivery of protein and peptide drugs when formulated into nanoparticles (Allemann et al., 1998; Birrenbach and Speiser, 1976; Couvreur and Vauthier, 2006; Vauthier et al., 2003; Vranckx et al., 1996). PACA nanoparticles can be prepared by a one-step *in situ* interfacial polymerization technique using a w/o-microemulsion as a polymerization template (Pitaksuteepong et al., 2002; Watnasirichaikul et al., 2000). Previously, it was known that the polymerization reaction can be initiated by various initiator molecules, including water, alcohols or weak bases, such as amino acids (Leonard et al., 1966; Vauthier et al., 2003). The initiation step can either involve an anion, such as OH^- (anionic polymerization), or a zwitter-ion, such as an amino acid (zwitter-ionic polymerization). The propagation reaction, however, always involves the addition of an anionic chain end to a Michael-acceptor monomer (Robello et al., 1999). Generally, it was believed that the abundant OH^- ions in water were the most likely nucleophilic reaction initiators. The major role of OH^- ions in the polymerization process of PACA was confirmed in several studies using IR spectroscopy (Aboubakar et al., 1999; Costa et al., 1997;

Leonard et al., 1966) and MALDI TOF MS (Weyermann et al., 2004). It could be shown that an OH-group was integrated permanently into the structure of the polymer chains (presence of an OH group signal at 3600 cm^{-1} in the IR spectrum). The term "initiator molecule" is used ambiguously in the literature, and can refer to molecules being permanently integrated as the starter molecule of the polymer chain (Costa et al., 1997; Kafka et al., 2009) or to molecules that could be regarded as catalysts and leave the polymer after initiation due to thermodynamic instability of the resulting oligomer chains (Robello et al., 1999). The *in situ* interfacial polymerization technique requires both the monomer and the drug to be present during the polymerization process. This gives the drug an opportunity to covalently interfere in the initiation and polymerization process. The presence or absence of an OH signal at the 3600 cm^{-1} band in the IR spectra of biopharmaceuticals was taken as the sole indicator for interference of drug in the initiation process (Aboubakar et al., 1999).

Subsequently, many other analytical techniques were introduced and complemented IR spectroscopy for the characterization of drug-polymer interactions. Chromatographic methods dominated PACA polymer analysis, together with NMR (Guise et al., 1990; Layre et al., 2006; Page-Clisson et al., 1998), MS (Hillery et al., 1996; Weyermann et al., 2004) and DSC (Sullivan and Birkinshaw, 2004). The identification of chemical modifications of polymer fractions through chromatography, i.e., by the occurrence of additional bands/peaks (Behan and Birkinshaw, 2001; Fawaz et al., 1997; Grangier et al., 1991; Guise et al., 1990; Ryan and McCann, 1996), however, has a number of inherent problems. Size exclusion chromatography (SEC) for example, separates the analytes according to a bulk characteristic: the hydrodynamic volume. The detection of signals based on the refractive index or UV absorbance of eluents in SEC is non-selective and molecular species co-eluting at the same time contribute to the same signal. Unambiguous identification of modified species is not possible. In order to interpret a chromatogram, reference chromatograms are required and, depending on the preparation of reference samples, physical mixtures of preformed polymeric nanoparticles and drug may still retain the potential to interact whilst suspended in the liquid phase (Kafka et al., 2009). The assumption that no peptide–polymer interactions can occur in a physical mixture may lead to misinterpretations. A laser desorption/ionization technique in combination with mass spectrometry was used first by Hillery et al. (1996) to identify covalent conjugation of drug with PACA polymer. These authors detected a mass shift of the polymer series when polymerized in the presence of a gonadotropin-releasing hormone (GnRH)-derivative (Hillery et al., 1996). However, no fragmentation was carried out to confirm that the conjugation of peptide caused the mass shift, thus unambiguous evidence for the covalent association of polymer with peptide was missing.

Recently, our research group has utilized MALDI TOF/TOF mass spectrometry coupled with CID to unambiguously identify permanent covalent conjugation of the bioactive neuropeptide D-Lys^6-GnRH to poly(ethylcyanoacrylate) (PECA) nanoparticles (Kafka et al., 2009). *In situ* loaded and polymerized PECA nanoparticles were washed in ethanol, dissolved in approximately 20–40 μl acetonitrile. 1.0 μl of sample solution was mixed with 9.0 μl of matrix (10 mg/ml CHCA dissolved in 60% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA). Those conditions were found to be suitable for the favorable and simultaneous detection of peptide and peptide-associated polymer. The sample was transferred by the one-layer, dried-droplet method and allowed to air-dry. Samples were analyzed on a 4800 MALDI tandem Time-of-Flight Analyzer (Applied Biosystems, MA, USA). Precursor ions of interest were selected for collision-induced dissociation tandem mass spectrometry (CID-MS/MS). CID-MS/MS spectra were acquired with 2000–4000 laser pulses per selected precursor using the 2 kV acceleration potential and air as the collision gas at a pressure of 1×10^{-6} torr. CID-MS/MS spectra were interpreted manually. Only b-, y- and a-type ions as well as relevant immonium ions were considered and annotated according to the Biemann nomenclature (Biemann, 1990).

The spectra for unloaded PECA nanoparticles have a characteristic molecular weight distribution of polymer and are distinct from the spectra for PECA nanoparticles *in situ* loaded with D-Lys^6-GnRH (Fig. 3). The characteristic peak increments of 125 mass units correlated with the sequential addition of ECA monomer subunits (Fig. 3a). The peak series ranged from $m/z < 1000$ to >6000, indicating a broad molecular weight distribution of PECA oligomer/polymer chains with polymers >40 monomer subunits detectable. The strongest signals were obtained for PECA 16- to 20-mers (m/z 2000–2500). The situation was different when D-Lys^6-GnRH was present during the *in situ* polymerization process. The start of a peptide-associated peak series with peak increments of 125 mass units can be observed at m/z 1378.7 (Fig. 3b). The free peptide showed a signal $[M+H]^+$ at m/z 1253.7, whilst all peptide/ECA conjugates are shifted by mass increments of 125 mass units $[M+(125)_n+H]^+$ (Fig. 3b). The strongest signals were obtained for peptide associated with 2–4 ECA subunits (m/z 1503.8, 1628.8 and 1753.8) (Fig. 3b). Non-peptide-associated oligomers/polymers were hardly visible in the low molecular mass range (m/z 600–1200) (Fig. 3b). This may be due to ion suppression effects, which were described earlier in Section 3 as a limitation of

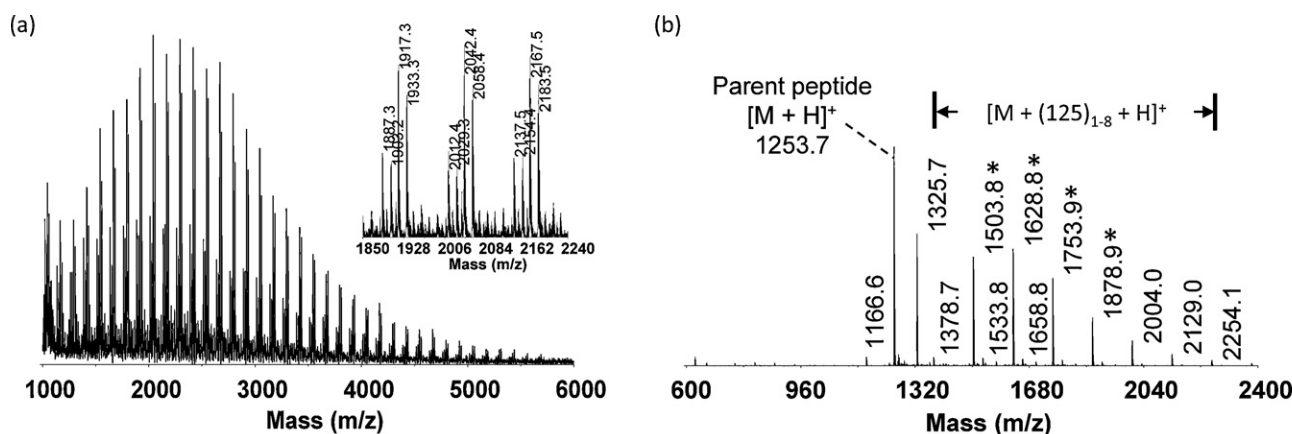


Fig. 3. Mass spectra of (a) unloaded PECA nanoparticles (b) PECA nanoparticles polymerized in the presence of D-Lys^6-GnRH. The insert to (a) shows an enlarged fragment of the mass spectrum. (b) Free peptide $[M+H]^+$ is visible at m/z 1253.7 and peptide–ECA conjugates $[M+(125)_n+H]^+$ at m/z 1378.7, 1503.8, 1628.8, 1753.9, 1878.9, 2003.0 etc. Precursor ions (*) were selected for CID fragmentation.

MALDI TOF MS. However, this particular case demonstrates how a limitation can also become an advantage.

CID fragmentation of selected peptide/ECA-conjugates precursor ions (*) revealed detailed information on the specific site of oligomer/polymer attachment to the peptide molecule. The histidine residue (His) was unambiguously identified as the site of modification (Fig. 4) (Kafka et al., 2009). In Fig. 4, the CID-MS/MS spectra of the unmodified parent peptide $[M+H]^+$ (precursor *m*/*z* 1253.7) (a) was aligned with CID-MS/MS spectra obtained for peptide associated with 2–5 ECA subunits $[M+(125)_{2-5}+H]^+$ (precursor *m*/*z* 1503.8, 1628.8, 1753.8 and 1878.8). All b-ions > b_2 were shifted by a multiple of 125 mass increments, suggesting position 2 of the D-Lys[6]-GnRH molecule (His) carries the ECA adducts. Furthermore, the diagnostic His immonium ion (i-His) at *m*/*z* 110.1 (Fig. 4a) was also shifted by a multiple of 125 mass increments, identifying His as the site of attachment. The b_1 ion and the y_2–y_8 ions were consistent in all precursor spectra. No other residues were found to be modified. This study demonstrates how, in particular, CID fragmentation can assist the unambiguous identification of the specific site of modification within the molecule, unraveling valuable information about the reaction mechanism. From this study, the reaction mechanism, involving the His residue as a nucleophilic initiator of a zwitter-ionic reaction pathway was proposed (Kafka et al., 2009). Interestingly, the *ex situ* addition of peptide to pre-formed PECA nanoparticles also yielded peptide–polymer conjugates, indicating the ability of the polymer to re-enter the equilibrium stage and provide reactive polymer species (Kafka et al., 2009). Lowering the pH to 1.9, which should have decreased the nucleophilicity of the imidazole ring of His (pK_b 7.96), did not circumvent the conjugation reaction (Kafka et al., 2010). These studies demonstrate the inherent high reactivity of D-Lys[6]-GnRH towards the polymer PECA and also the limited control of this reaction by the formulator. Thus, the formulation of small histidine-containing peptides, such as D-Lys[6]-GnRH, into PACA nanoparticles is an example for a pharmaceutical formulation with limited perspective to enter the biopharmaceutical market for human therapeutics.

4.2. In situ synthesis of BSA–polymer conjugates via RAFT polymerization

Polymer systems, including PACA nanoparticles, have also been deliberately conjugated to protein and peptide bioactives for reasons of enhanced entrapment efficiency (Liang et al., 2008), modified/sustained release characteristics (Grangier et al., 1991; Singh et al., 2007), targeted release (Pasut and Veronese, 2007), improved stability (Hillery et al., 1996; Pasut and Veronese, 2007), prolonged biological half lives (Pasut and Veronese, 2007) and enhanced absorption (Veronese and Harris, 2008). This approach, however, requires the site-specific functionalization of either protein/peptide or polymer in order to produce hybrid constructs with well-defined protein/peptide segments and well defined polymer chains (Gauthier and Klok, 2008). In an excellent review, Gauthier and Klok (2008) have summarized the recent advances in the field to produce such well-defined hybrid conjugates, including the functionalization of proteins, peptides and polymers, and strategies to assemble the protein/peptide–polymer hybrids. The key strategy in all the reactions described, however, is that by functionalizing the bioactive or polymer before polymerization, very specific reactions can be initiated in a controlled way. Herein, we describe one example of a divergent strategy described by Boyer et al. (2007) to produce hybrid conjugates of the protein bovine serum albumin (BSA) and the polymer poly(*n*-isopropylacrylamide) (poly(NIPAAm)) using a modified reversible addition-fragmentation chain transfer (RAFT) technique.

RAFT is one controlled radical polymerization (CRP) technique that is used to minimize the heterogeneity of protein/peptide–polymer conjugates. Previously, using RAFT it was possible to synthesize well-defined end-group-functionalized polymers, however, the polymers reacted with the protein in a relatively uncontrolled stoichiometry with limited control over the number of polymer conjugations per protein molecule. In order to improve control of the conjugation reaction, Boyer et al. (2007) synthesized a BSA-bound macroRAFT agent, which reacts with monomer via a free radical mechanism (Fig. 5). The free radical polymerization reaction was initiated by the addition of a chemical initiator (4,4′-azobis[2-9-imidazolin-2-ethyl)propane] dihydrochloride) instead of gamma-radiation, in order to preserve the integrity of the protein.

The successful conjugation of bovine serum albumin (BSA) to the RAFT agent to produce the BSA-macroRAFT agent was confirmed using UV/VIS spectroscopy, MALDI TOF MS (Fig. 6) and H^1 NMR. For their MALDI TOF MS analysis, these authors used SA (10 mg/ml) as matrix in a sample to matrix ratio of 1:1 (v/v). The mixture was transferred by the one-layer, dried-droplet method and analyzed on a Voyager STR TOF mass spectrometer (Perspective Biosciences), which was operated in linear ion mode. They found an average mass shift of 1200 mass units, caused by the addition of one RAFT agent molecule to BSA (Fig. 6). The broadening of the signal observed following the conjugation was explained by the polydispersity inherent in the raw materials used (PDI of the polyethylene glycol (PEG)-based RAFT agent was 1.06, which was explained by the polydispersity of the PEG raw material used in the study). BSA was chosen as a model protein for this study on the basis of providing only one potential SH-group at the Cys^{34} for the conjugation reaction. The authors did not record any fragment spectra to confirm the specific site of conjugation is the SH-group of BSA. This could be attributed to the fact that, considering the size of the molecules involved, the conjugate would have to be digested before it could be fragmented, which might have not been a feasible approach. Instead, they used complimentary methods, such as NMR and a wet-chemical assay (Ellman's assay) to determine the content of free SH-groups, to confirm that the conjugation took place exclusively at the SH-group of Cys^{34}.

The final product of the free radical-initiated *in situ* polymerization of NIPAAm following the conjugation to the BSA-macroRAFT agent was also confirmed using MALDI TOF MS. Although the mass spectra were not presented in the article, the authors observed BSA-macroRAFT conjugate signals at *m*/*z* 80,100 and 102,900, which corresponded to 10 and 20% conversion of monomer, respectively. Signals in the higher mass range, corresponding to higher conversion rates of monomer could no longer be resolved. Although all spectra were acquired in the linear ion mode, molecular weights of 100 kDa and higher must be considered a mass range exceeding the limits of many MALDI TOF mass spectrometers. Again, digestion prior to mass spectrometric analysis would be required. The complementary methods of NMR and SEC were employed to confirm the molecular weights of the BSA-macroRAFT conjugates with 10 and 20% conversion rates as determined by MALDI TOF MS (Boyer et al., 2007).

4.3. Poly(lactic-co-glycolic acid) (PLGA) in biopharmaceuticals

A considerable amount of research in the biopharmaceutical field focuses on the use of preformed poly(lactic-*co*-glycolic acids) (PLGA) for drug carriers, since they are FDA-approved and appeal for a number of reasons (Lu et al., 2009; Mishra et al., 2010). However, they are also known to potentially affect the stability of protein and peptide bioactives by forming an acidic microenvironment (as low as pH 2) inside the formulation (Panyam and Labhasetwar, 2003; Park et al., 1995; Shao and Bailey, 1999; Uchida

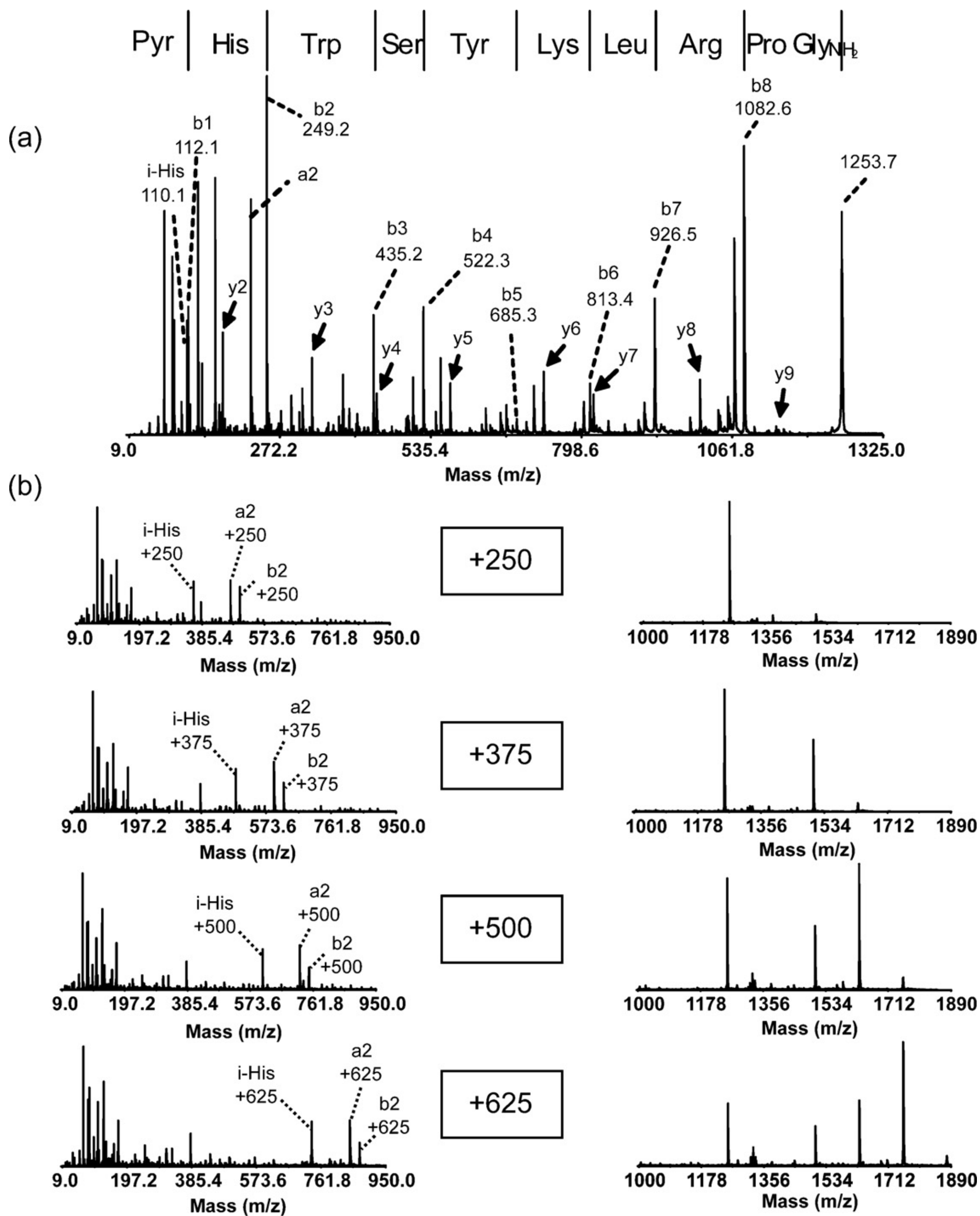


Fig. 4. MALDI TOF MS/MS spectra of (a) the parent peptide D-Lys^6-GnRH (m/z 1253.7), fragments aligned with the amino acid sequence of the D-Lys^6-GnRH and (b) a sequence of four precursor ions (*) of the peptide–polymer conjugates (top to bottom m/z 1503.8, 1628.8, 1753.8 and 1878.8); b_1–b_8 fragments carry the N-terminus, y_2–y_9 fragments carry the C-terminus of the D-Lys^6-GnRH. Left: enlargement of the low molecular range; the diagnostic b_2 and a_2-fragment ions, and the histidine immonium ion (i-His) are indicated and shifted by mass units of $(125)_n$ with n referring to the number of monomer subunits conjugated to peptide (top to bottom 2, 3, 4, 5). Right: enlargement of the higher molecular range showing fragmentation of the precursor ions (*) with increments of m/z 125; precursor m/z 1503.8 shows fragment signals at m/z 1378.8 and 1253.7, precursor 1628.8 at m/z 1503.8, 1378.8 and 1253.7, precursor 1753.8 at m/z 1628.8, 1503.8, 1378.8, 1253.6 etc.
Reproduced from Kafka et al. (2009), with permission from the American Chemical Society.

et al., 1996) and to modify protein bioactives by way of acylation during the degradation process of the polymer (Houchin and Topp, 2008; Na et al., 2003). Such processes are unwanted and could alter bioactivity and toxicity of the bioactive compound (Na et al., 2003). According to ICH guidelines (enacted by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use), acylation products of bioactives should be considered a drug-derived impurity and investigated for toxicity and adverse pharmacological effects (Na et al., 2003, 2007). In this case study, Na et al. (2003) prepared PLGA microspheres by the solvent extraction/evaporation method. Peptides investigated in this study included salmon calcitonin (sCT) and human parathyroid hormone 1-34 (hPTH 1-34), both containing primary amino groups (N-terminus and lysine residues) as well as OH-groups (tyrosine or serine); and leuprolide, a GnRH agonist, which only contains OH-groups in tyrosine and serine residues. The resulting microspheres were incubated at 37 °C for 28 days in phosphate buffered saline (pH 7.4) containing 0.02% Tween 80 and 0.02%

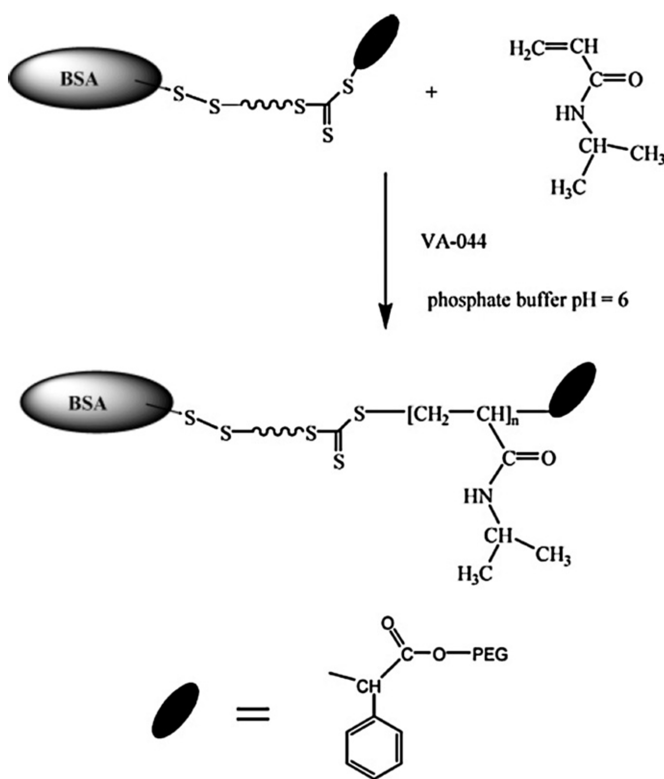


Fig. 5. Schematic representation illustrating the reaction of the BSA-macroRAFT agent with monomer. Reproduced from Boyer et al. (2007), with permission from the American Chemical Society.

sodium azide. For MALDI TOF MS analysis, CHCA (saturated solution in 50% acetonitrile containing 0.3% trifluoroacetic acid) was used as matrix. The samples were mixed with matrix in a ratio of 1:1 (v/v) and transferred by the one-layer, dried-droplet method. Samples were analyzed on a Voyager Biospectrometry Workstation (Perspective Biosystem, MA, USA). Spectra were acquired in linear ion mode. Spectra were smoothened (with a 19-point Savitzky-Golay filter).

All mass spectra recorded for the three peptides investigated in this study showed only one signal for the unmodified parent peptide $[M+H]^+$ at $t = 0$ days of incubation at m/z 3432.67 for sCT, m/z 4114.78 for hPTH(1–34) and m/z 1210.79 for leuprolide, respectively (Figs. 7 and 8). After 28 days of incubation at 37 °C, the spectra for the two peptides containing primary amines (sCT and hPTH(1–34)) were very similar. Additional signals $[M+(58)_n+H]^+$ could be observed in the mass spectra corresponding to glycolic acid acylation products of sCT and hPTH(1–34) (Fig. 7). For example, 1, 2 and 3 glycolic acid units were added to sCT after 7, 14 and 28 days respectively, as demonstrated by the occurrence of signals at m/z 3491.26 $[M+(58)_1+H]^+$, m/z 3549.42 $[M+(58)_2+H]^+$ and m/z 3607.82 $[M+(58)_3+H]^+$ (Fig. 7b).

In contrast, leuprolide, in the absence of primary amines, did not show any signs of chemical modification following the incubation of peptide in phosphate buffer saline after 28 days (Fig. 8). This study indicates that the tendency of peptide to be chemically modified via acylation as a result of PLGA degradation is highly dependent on the chemical composition of the bioactive. Using MALDI TOF MS, Na et al. (2003) revealed valuable information on the mechanism of acylation of proteins and peptides formulated with PLGA. The acylation mechanism appears to involve the amidation of primary amines rather than the esterification of OH-groups. However, a CID fragmentation was not possible with the instrument used to confirm the site of modification within the peptide molecules.

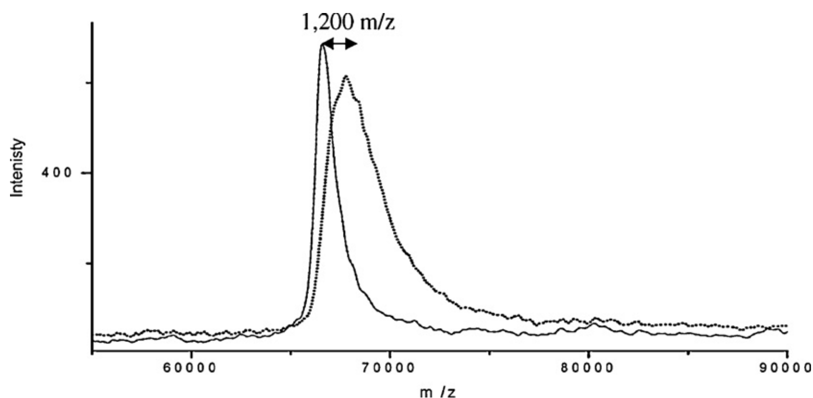


Fig. 6. MALDI TOF mass spectrum obtained for BSA before (m/z 66,400) and after conjugation to the RAFT agent (m/z 67,600). The mass shift corresponds to the mass of one RAFT agent molecule (m/z 1200). Reproduced from Boyer et al. (2007), with permission from the American Chemical Society.

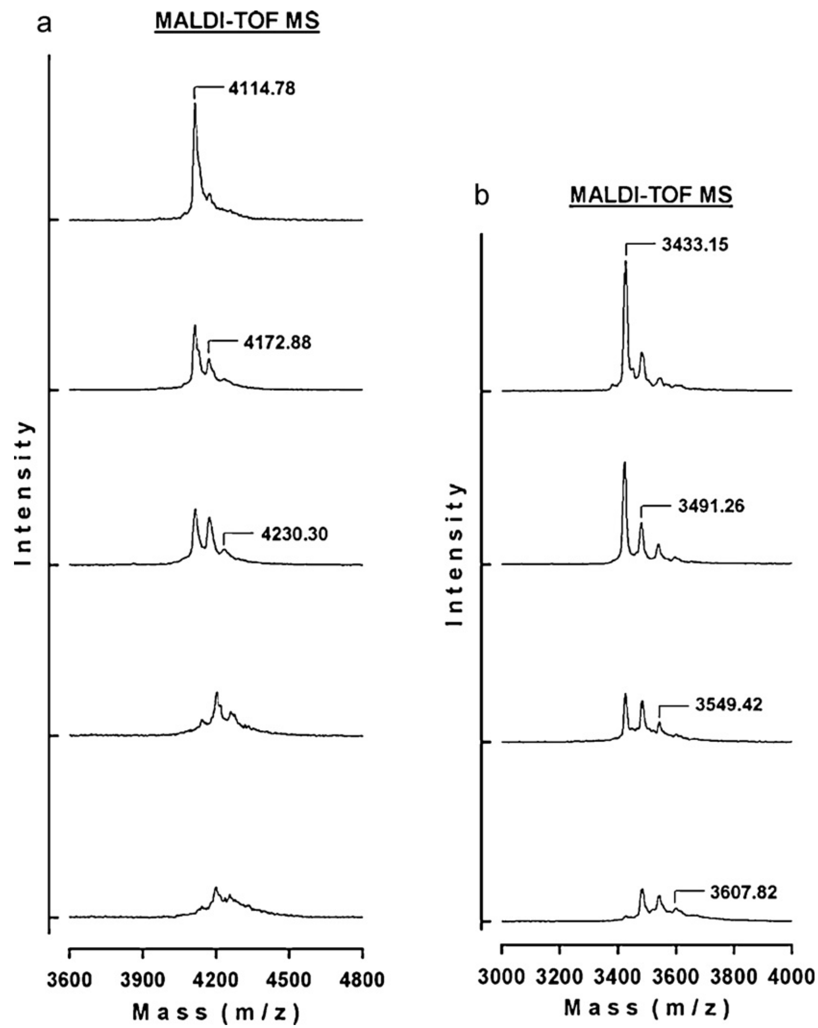


Fig. 7. Mass spectra of (a) hPTH(1–34) and (b) sCT contained in PLGA microspheres and monitored during incubation in phosphate buffer saline pH 7.4 at 37 °C for 28 days. (a) Unmodified peptide $[M+H]^+$ is visible at m/z 4114.78.
Reproduced from Na et al. (2003), with permission from the Controlled Release Society.

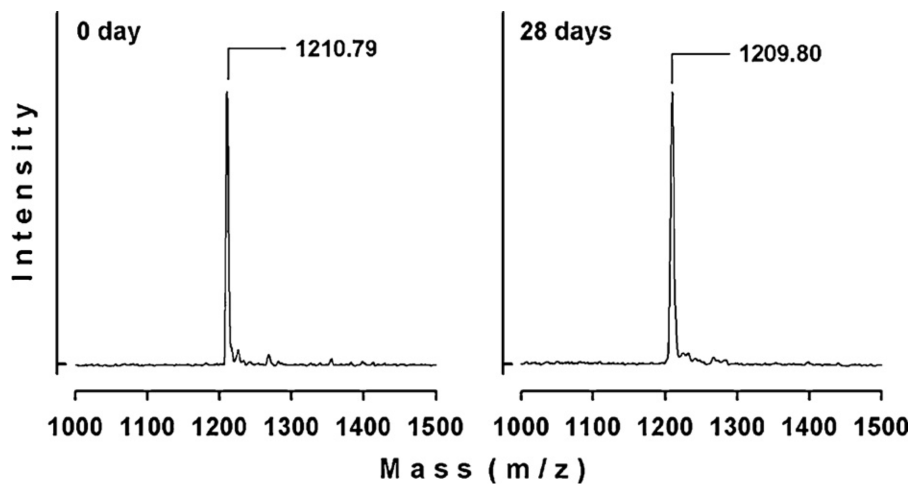


Fig. 8. Mass spectra of leuprolide in PLGA microspheres (a) before and (b) after incubation in phosphate buffer saline pH 7.4 at 37 °C for 28 days. Unmodified peptide $[M+H]^+$ is visible at m/z 1210.79. Reproduced from Na et al. (2003), with permission from the Controlled Release Society.

5. Conclusions and outlook

We have outlined the variety of parameters influencing the performance of MALDI TOF MS and how they can be adapted to analyze a wide range of analytes. MALDI TOF MS/MS provides a unique combination of accurate mass measurements of intact biomolecules ranging from 1 to 100 kDa, high throughput analysis, and the opportunity to reveal detailed, structural information on the molecular level via ion fragmentation for small sample sizes (femtomoles) in usually less than 5 min. MALDI TOF MS is expected to gain more importance as a very efficient tool for the analysis and characterization of formulations containing biopharmaceutical compounds. The application of MALDI TOF MS for the analysis of protein/peptide–polymer conjugates, however, demonstrates that every technique has limitations and that the application of complementary and orthogonal methods is necessary for comprehensive analysis of modern biopharmaceutics.

The opportunities through further optimization of parameters, discovery of suitable matrices etc. seem endless and MALDI TOF MS continuously extends its applications into new directions. The application of MALDI (TOF) MS as a recent imaging technology, for example, is highly regarded in all life sciences (Francese et al., 2009) and particularly important as an integral component of modern drug discovery and development (Solon et al., 2010) or process analytical technology (PAT) (Earnshaw et al., 2010).

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