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Investigation of Protein–Ligand Interactions by Mass Spectrometry

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The rate of drug discovery is greatly dependent on the development and improvement of rapid and reliable analytical methods that allow screening for protein–ligand interactions. The solution-based methods for investigating protein–ligand interactions by mass spectrometry (MS), which are discussed in this paper, are hydrogen/deuterium exchange of protein backbone amide hydrogens, and photoaffinity labeling. Moreover, MS analysis of intact noncovalent protein–ligand complexes is described. Fourier transform ion cyclotron resonance mass spectrometry (FTICR–MS) with its ultra-high resolution and excellent mass accuracy is also considered herein as it is gaining increasing popularity for a mass spectrometric investigation of protein–ligand interactions.

1. Introduction

Modern drug discovery relies on the structural analysis of proteins and the complexes they are forming with various target molecules. Among the great variety of protein–ligand interactions are protein–protein, protein–nucleic acid (DNA, RNA), protein–cofactor, protein–metal, and protein–drug interactions. A characteristic feature of interactions between proteins and their binding partners is the transient nature of the interactions. In case a 1:1 complex is formed between a protein (P) and a ligand (L), a dynamic equilibrium exists between the protein–ligand complex (PL) and its components [Eq. (1)]:

$$P + L \rightleftharpoons PL \quad (1)$$

The dissociation and association constants (K_d and K_a) are defined as [Eq. (2) and (3)]:

$$K_d = [P]\,[L]/[PL] \quad (2)$$

$$K_a = 1/K_d \quad (3)$$

There are a variety of analytical techniques available to detect the presence of a protein–ligand interaction and to determine its dissociation constant K_d. In pharmaceutical research, emphasis in screening for protein–ligand interactions is increasingly placed on high-throughput capabilities, taking advantage of the inherent advantages of mass spectrometry (MS), namely high speed and high sensitivity. Ongoing rapid instrumental development makes MS an important technique for analyzing protein–ligand interactions. MS is unique in that it is capable of detecting and characterizing individual conformational states of a protein that may coexist in equilibrium in the solution. As a result of its superior sensitivity, MS uses only minute amounts of proteins—down to the subfemtomolar level—for analysis, which allows proteins to be studied at endogenous levels, which is one of its great advantages compared to other techniques such as NMR spectroscopy. The solution-based methods for investigating protein–ligand interactions by mass spectrometry that will be discussed in this paper are hydrogen/deuterium exchange (HDX) of protein backbone amide hydrogens[1] and photoaffinity labeling.[2] Moreover, the analysis of intact noncovalent protein–ligand complexes in the gas phase by electrospray ionization mass spectrometry (ESI–MS) will be described. Fourier transform ion cyclotron resonance mass spectrometry (FTICR–MS) is becoming more and more popular in drug discovery because of its excellent mass accuracy, and its ultra-high resolution enables more information to be obtained in a single measurement.[3] A novel hybrid mass spectrometer combining a linear ion trap and an orbitrap analyzer is likely to play an important role in future protein analysis as it offers high mass resolution, high mass accuracy, and a good dynamic range.[4] This article is by no means intended to be an exhaustive review on the various MS methods, which have become available for analyzing the diverse interactions between proteins and ligands, but the present paper aims to give a brief overview of existing MS-based approaches for analysis of protein–ligand complexes. A number of review articles have been published dealing with the different MS techniques that are used for investigating protein–ligand interactions, and the interested reader is encouraged to dig deeper into the already existing literature.[1,3,5–8]

2. Mass Spectrometry

2.1. The "Soft" Ionization Methods—MALDI (Matrix-Assisted Laser Desorption/Ionization) and ESI (Electrospray Ionization)

The development of mass spectrometry has always been closely linked to the development of novel ionization techniques, allowing analysis of polar and ionic molecules with increasing

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molecular mass. The soft ionization methods MALDI[9,10] and ESI[11] are the dominating MS ionization methods for analyzing biological macromolecules and thus, for characterizing protein–ligand interactions.

MALDI uses a specific matrix consisting of small organic compounds, such as derivatives of cinnamic acid or benzoic acid, which exhibit a strong resonance absorption at laser wavelength, typically 337 nm for UV-MALDI. The mechanisms of ion formation in MALDI are still a subject of continuing research.[12–14] MALDI generated a great demand for a mass analyzer ideally suited to be used in conjunction with a pulsed ion source, such as the time-of-flight (TOF) analyzer. The performance of TOF instruments has increased tremendously during the past few years and to date, two tandem TOF instruments are commercially available, which allow sequence information to be obtained by MS/MS (tandem MS) measurements.[15]

In ESI, liquids are sprayed in the presence of a strong electric field forming small, highly charged droplets. ESI requires a sample that is devoid of nonvolatile salts and detergents to obtain the highest sensitivity and a careful optimization of electrospray conditions for the specific compound under investigation. Miniaturization of the electrospray technique (nanoelectrospray), by applying narrower spray capillaries, results in smaller droplets, reduced flow rates, and improved sensitivity.[16,17] The peptide mixture is usually introduced into the mass spectrometer by a separation technique such as liquid chromatography (LC) or capillary electrophoresis (CE). Complex peptide mixtures are mostly separated by reversed-phase high-performance liquid chromatography (RP-HPLC).

In the pharmaceutical industry, MS measurements of small molecule drugs are typically performed using LC/ESI–MS/MS, and MS-based methods have been used for measuring the conversion of substrates to products in enzyme assays.[18] One great advantage of using an MS-based approach is the direct nature of the measurement, as a mass shift results from conversion of substrate to product. Thus, false positive and false negative results are minimized, which affect many of the traditional assays that are based on fluorescence, chemiluminescence, or radioactivity measurements.[19]

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For many enzymes with peptide substrates, ESI–MS based approaches have an added complication of dividing the substrate and product into multiple peaks because of charge state distribution, thus complicating data analysis. On the other hand, MALDI produces mainly singly charged ions, which simplifies data analysis as no data deconvolution is required. Thus, MALDI–TOF–MS offers a label-free and direct read-out of substrate and product for measuring the conversion of substrates to products in enzyme assays and compound screenings.[19] The reproducibility and quantitative capabilities of MALDI have been questioned compared to ESI-based methods. However, a growing number of publications have indicated that MALDI–TOF–MS based approaches can be employed for quantitative measurements.[20,21] MALDI allows data obtainment at a fast sampling rate, and it is tolerant to many buffer salts and reagents, which are present in enzyme assays. MALDI–TOF–MS has been employed to directly measure ratios of substrates and products based on MS signal intensities or peak areas to yield IC_{50} value curves for rapid inhibitor screening.[19] Currently, the sampling rate of modern MALDI mass spectrometers is approximately 10 seconds per sample, thus allowing over 7500 samples per day to be measured. The speed, sensitivity, low costs, and reproducibility of using MALDI–TOF–MS as a read-out system for enzyme assays makes it an alternative assay to determine dose-response curves for comparative IC_{50} measurements.

2.2. Fourier Transform Ion Cyclotron Resonance (FTICR) Mass Spectrometry

Among all mass spectrometric analyzers, the ion cyclotron resonance (ICR) principle offers the highest resolution and mass measurement accuracy.[22] Since its introduction in 1974,[23,24] there have been a number of studies demonstrating these capabilities. Modern, commercially available FTICR mass spectrometers offer a resolving power above 100 000 and mass measurement accuracies below 2 ppm on a routine basis. The principle underlying FTICR mass spectrometry forms the basis of its unique capabilities. There have been a couple of excellent reviews[25–28] that give very detailed descriptions of this technique, therefore only the very fundamentals are briefly described herein. FTMS uses the ICR principle to determine *m/z* values of ions, whereby ions are trapped in a cell that is located within a static magnetic field (Figure 1). Under the influence of the magnetic field, the ions describe a circular motion caused by the Lorentz force and the centrifugal force (inertia) affecting them in opposite directions. Hereby, the angular frequency ω_c of the so-called cyclotron motion depends solely on the *m/z* value of the circulating ions and the magnetic field strength B [Eq. (4)]:

$$\omega_c = B z/m \quad (4)$$

To measure the cyclotron frequency ω_c it is necessary to force the ions of one *m/z* type into coherent motion by applying a frequency pulse through the two opposite excitation electrodes, which is in resonance with the cyclotron frequency

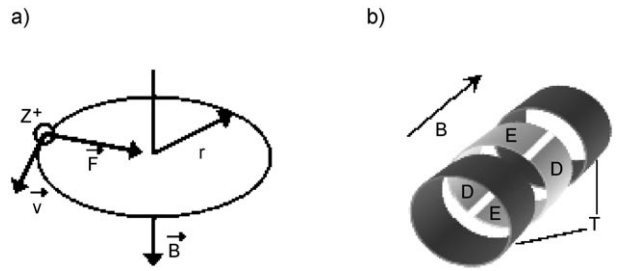


Figure 1. a) Force and resulting motion of an ion with the charge z+ in a magnetic field B. $\vec{v}$ is the velocity of the ion perpendicular to the direction of the magnetic field and r is the radius of the circular motion, $\vec{F}$ is the Lorentz force. b) Schematic setup of an ICR analyzer cell. E: excitation electrodes, D: detection electrodes, and T: trapping electrodes. The direction of the magnetic field B is indicated.

of the ions. As an additional result, the kinetic energy of the ions and therefore the radius of the circular motion will increase. After excitation, the circulating ion clouds induce an oscillating image current in the two opposite detection plates, whose frequency corresponds to the cyclotron frequencies and thus, to the *m/z* values of the ions. A breakthrough in the ICR technique was achieved when Comisarow and Marshall applied inductive detection and Fourier transformation to determine the cyclotron frequencies of all ions in the cell after simultaneous excitation by a broadband rf-pulse.[23] The image currents are recorded as a time domain signal, the so-called transient, and after amplification, digitization, and Fourier transformation, a frequency spectrum is obtained, which is easily converted into a mass spectrum.

Another aspect of the ICR technique is that this type of measurement is nondestructive; ions can be stored in the analyzer cell for several minutes or even hours. It is possible to study gas-phase reactions in the cell[29] or to fragment ions by various techniques, such as IRMPD[30] (infrared multi-photon dissociation), SORI-CID[31] (sustained off-resonance irradiation collision-induced dissociation), or ECD[32] (electron capture dissociation) during MS^n experiments.

FTICR–MS is gaining popularity for analyzing proteins as its excellent mass accuracy and its ultra-high resolution are advantageous for obtaining more information in a single measurement, especially when complex mixtures are investigated. Examples of using ESI–FTICR–MS in the drug discovery process include a report on measuring binding constants between aminoglycoside antibiotics and the decoding region of prokaryotic 16 rRNA.[33] Moreover, an FTICR mass spectrometry-based approach for small molecule drugs that act by binding to structured regions of RNA has been developed and termed multi-target affinity/specificity screening (MASS).[34,35]

3. Characterization of Intact Noncovalent Complexes

ESI–MS has gained outstanding importance for analysis of noncovalent complexes.[7,36–39] From measuring the molecular weights of the intact complex and those of the individual binding partners, the stoichiometry of the complex under investigation can be derived. Dissociation constants ranging from nM to mM can be deduced from the ESI mass spectra.[40,41] The types of biological systems that have been studied in the past few years using ESI–MS comprise a wide range of structurally different substances.[7,37–39] In contrast to ESI–MS, MALDI–MS has not gained general applicability for studying noncovalent complexes. There are, however, a few studies demonstrating that MALDI–MS can be employed to study noncovalently bound complexes if special precautions are taken in preparing the samples, applying suitable matrices, and avoiding harsh, acidic pH conditions.[42,43] It has also been observed that only spectra recorded from the top layer of the samples show pronounced signals of noncovalent complexes under such conditions (first shot phenomenon).[42]

The most important reason for the popularity of ESI–MS in the investigation of noncovalent complexes relates to its connection with the solution phase. For an ESI–MS analysis, a biochemical sample is introduced into the mass spectrometer and transferred in the gas phase. Although it is of interest for some biophysical studies to study the behavior of biological molecules in a solvent-free environment, most biological questions are answered based on the properties of these molecules in the solution. Some fundamental questions arise:

- Can the measurement of a molecule in the gas phase be employed to derive the original properties of the respective molecule in the solution?
- How close do gas phase and solution phase structures resemble each other?

The nature of the specific interaction playing a role for noncovalent binding behavior in the solution can be discriminated from each other by ESI mass spectrometric measurements in the gas phase. Electrostatic interactions are enhanced in a solvent-free environment, thus making complexes based on electrostatic interactions extremely stable in the gas phase.[44,45] On the other hand, interactions that are based on hydrophobic interactions are weakened in vacuo.[46] The different kinds of gas phase interactions have to be considered to draw meaningful conclusions concerning the relative and absolute binding constants of molecules in solution.

As ESI–MS is highly selective, it is possible to screen multiple compounds simultaneously for binding to a target protein. Figure 2 shows an example of such an assay, in which a mixture of saccharides was screened for binding to lysozyme.[5,47] The mass spectrum obtained after mixing the protein with six test compounds clearly shows that only one of the saccharides (*N,N′,N″*-triacetylchitotriose (NAG_3)) possesses a high affinity for lysozyme. None of the other saccharides are known to bind to the protein, which is consistent with the mass spectrum exhibiting only low-intensity peaks of those ligands with lysozyme.

4. H/D Exchange

Protein hydrogen/deuterium exchange (HDX) employs the relationship between isotopic exchange rates of main chain amide hydrogens in proteins and their secondary and tertiary struc-

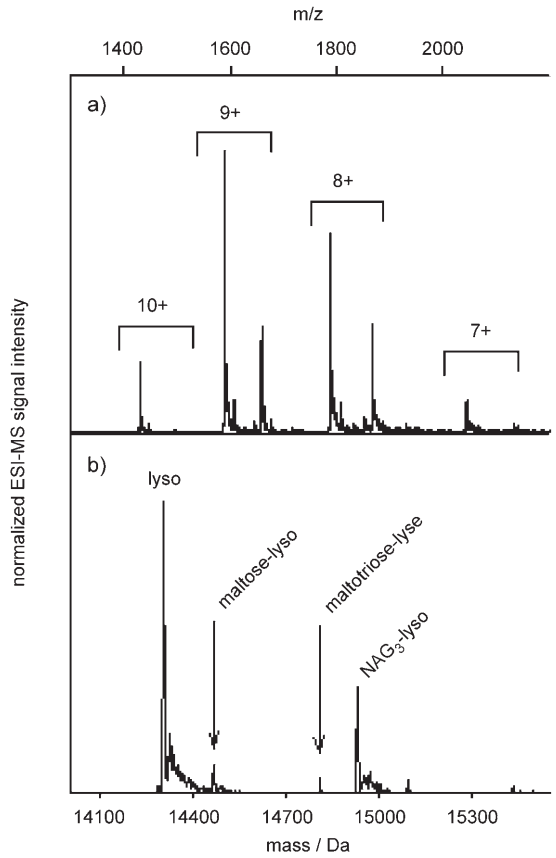


Figure 2. Direct ESI–MS screening of a compound mixture binding to a protein, reprinted with permission from.[5] a) ESI mass spectrum of a 10 μM solution of lysozyme containing ribose, rhamnose, glucose, maltose, maltotriose, and *N*,*N*′,*N*″-triactylchitotriose (NAG_3) at concentrations of 10 mM each. The charge states are indicated. b) Deconvolution of the mass spectrum shown in a). Ions of noncovalent complexes involving lysozyme and individual saccharides are denoted with arrows.

tures. These amide hydrogens are distributed uniformly at every amino acid, except proline, in a protein molecule. The theory behind this technique is that the exchange rates of the amide protons are directly related to protein structure characteristics, namely the extent to which the protons are shielded from the solvent and whether they are involved in intramolecular hydrogen bonding.[48–52] Using ESI and MALDI mass spectrometry, H/D exchange rates are examined by determining the mass increases (Δm).[53,54] The rates of amide hydrogen exchange can be adjusted by simply altering pH and temperature. The exchange half-life for unprotected amide hydrogens is over one hour at a pH 2–3 and 0 °C, which allows the peptides to be analyzed by mass spectrometry.[52] During the exchange experiment, one takes aliquots at certain time points and subjects the protein to pH 2.5 and 0 °C to quench the exchange reaction. These conditions imply the use of a protease, which is active under acidic conditions, to cleave the protein before subsequent MS analysis. Therefore pepsin—a nonspecific protease—is usually employed for protein digestion. When using MALDI, the exchange process is quenched by adding acidified matrix solution followed by deposition on the MALDI target. However, great care must be taken to avoid further exchange (or back-exchange) during sample preparation and/or laser-induced desorption.[55,56]

In general, two types of amide exchange experiments are possible for studying protein–ligand interactions.[55] In the on-exchange experiment, the protein–ligand complex shows a region, in which less deuterium is incorporated compared to control experiments using the protein alone (Figure 3 a). In the off-exchange experiment, each protein is allowed to incorporate deuterium. After complex formation, the deuterium atoms are off-exchanged by dilution with H_2O. In the off-exchange experiment, the presence of remaining deuterium atoms indicates the interface after off-exchange as compared with control experiments using each binding partner alone (Figure 3 b).

Instead of digesting the proteins after H/D exchange before MS analysis is performed on the peptide mixtures, the proteins might be directly fragmented inside the mass spectrometer.[57,58] ESI–FTICR–MS has been successfully employed for the latter purpose, for example, to study the structural stability of myoglobin.[59] Another publication deals with the structural investigation of the interface within an enzyme–inhibitor complex.[60] The use of FTICR–MS in combination with H/D exchange additionally allows analysis of gas-phase structures, as protein ions can be stored in the analyzer before they are interrogated with gaseous D_2O. Using this method, different stable gas-phase conformers of bovine cytochrome *c* have been probed[61] and a study on bradykinins demonstrated the existence of peptide zwitterions in the gas phase.[62]

The SUPREX (stability of unpurified proteins from rates of H/D exchange) approach uses MALDI and measures the stability of proteins upon chemical denaturation in the absence and in the presence of one or a number of competing ligands.[63,64] This is achieved by monitoring the change in H/D exchange rates induced by addition of a denaturing agent, such as guanidinium chloride or urea. By denaturing the protein, parts of the protein originally buried become accessible, thus leading to a faster exchange of these amide backbone protons. The increase of mass (Δm) is plotted against the concentration of denaturing agent resulting in typically sigmoidal SUPREX curves. The transition from lower to higher Δm values reflects the transition from folded to unfolded protein as more amide protons become accessible for H/D exchange with increasing denaturant concentration.[5,65] SUPREX allows for determining the free energy values associated with protein unfolding reactions (ΔG_u).[66] When H/D exchange is employed for the evaluation of ΔG_u values, an assumption of so-called EX2 exchange behavior is required, which means that the protein refolding rate must be significantly faster than the intrinsic exchange rate K_{int} of an unprotected amide proton.

The abbreviation PLIMSTEX refers to: protein–ligand interactions in solution by mass spectrometry, titration, and H/D exchange.[67] This approach relies on an altered H/D exchange upon equilibrium titration of the protein with the ligand, which is monitored by *m/z* shifts in the mass spectrum. A PLIMSTEX profile displays the mass shift (Δm) resulting from amide H/D exchange as a function of ligand concentration.[5,65] The Δm values decrease with increasing ligand concentration as a result of an increased protection of the backbone amide

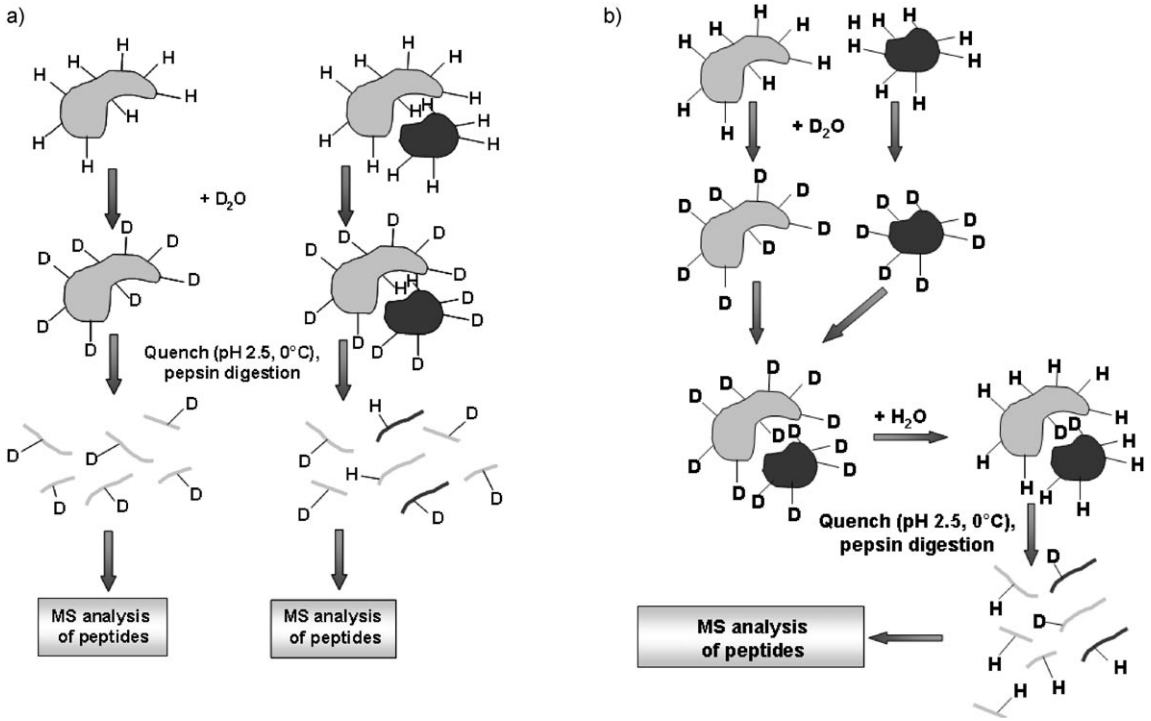
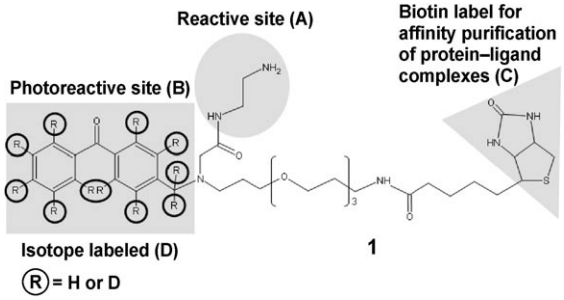


Figure 3. Scheme of the a) on-exchange and b) off-exchange experiment used for protein hydrogen/deuterium exchange.[55] The exchange reaction is quenched at pH 2.5, 0 °C before pepsin digestion and MS analysis is performed on the resulting peptide mixture.

hydrogens caused by formation of the protein–ligand complex. Very recently, PLIMSTEX has been adapted for determining protein–ligand affinities in solution, for determining self-association equilibrium constants for proteins, and for applying them to various insulin analogues.[68] This adaptation has been termed SIMSTEX (self-association interactions using mass spectrometry, self-titration, and H/D exchange). SIMSTEX has proven its usefulness for screening the oligomerization properties of newly developed insulin analogues, but is likely to also be valuable for screening the self-association of larger proteins.



Scheme 1. Structure of isotope-labeled photoaffinity reagent **1**[70] with the structural elements A. amine-reactive site, B. photoreactive site, C. biotin label, D. isotope label (black circles).

5. Photoaffinity Labeling

In photoaffinity labeling (PAL), a covalent linkage is created between a ligand and a protein upon irradiation by UV light. Reliable and reproducible high-efficiency labeling of target proteins is achieved by phenyl azides, diazirines, and benzophenone photophores.[69]

A novel photoaffinity label **1** (Scheme 1) has been presented recently by Lamos et al.,[70] which was termed Target-Identification Probe (TIP). **1** has been successfully employed to identify the interface region between the immunosuppressive drug cyclosporin A (CsA) with its target protein cyclophilin A (CypA) in the presence of the three nonbinding proteins ovalbumin, carbonic anhydrase, and FK binding protein (FKBP).[70] The employed strategy is schematically presented in Figure 4:[70,71] In the first step, the 1:1 mixture of nondeuterated and 11-times deuterated photoaffinity label **1** is coupled to the bioactive ligand CsA. After the coupling reaction, the conjugate is incubated with a protein mixture and the photoreaction is induced by irradiating the mixture with long-wavelength UV light. Only the target protein CypA, which specifically interacts with the ligand CsA, undergoes the photo-crosslinking reaction, whereas nonbinding proteins are not covalently attached to the ligand. Affinity chromatography using avidin beads is performed for purification of the protein–ligand complex created. The purified complex is enzymatically digested, for example, by trypsin, which cleaves proteins at the C-terminal site of lysine and arginine residues. ESI and/or MALDI mass spectrometry can be used to analyze the peptide mixtures created and MS/MS experiments are extremely valuable for obtaining sequence information about the proteolytic peptides derived from the target protein and for revealing the amino acids that have been modified by the photoaffinity labeling procedure. Applying the photoaffinity label in a fixed mixture of nondeu-

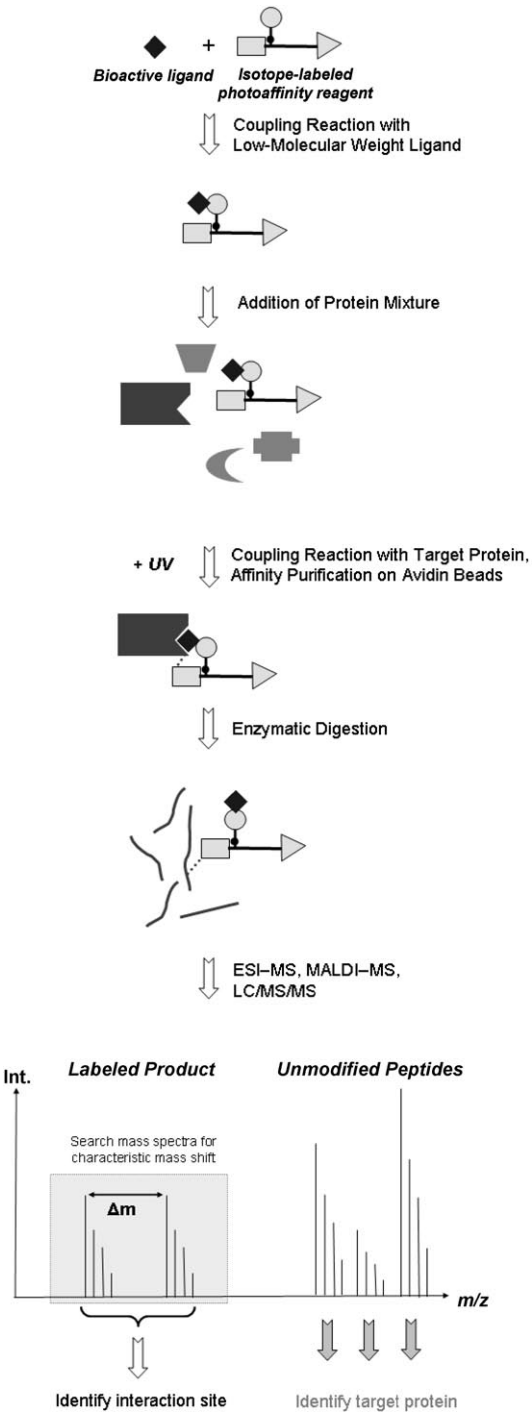


Figure 4. Strategy for analyzing protein–ligand interactions using photoaffinity label **1** in a 1:1 mixture of D_0 and D_{11} derivative, as presented in reference [70] for studying CypA–CsA interaction. The structural components of the photoaffinity label are schematically depicted according to Scheme 1. Reprinted from reference [71]. Copyright Wiley-VCH, 2007.

terated and deuterated derivative allows for a greatly facilitated MS identification of peptide–ligand adducts owing to the characteristic isotope patterns of the modified peptides. Signals exhibiting the characteristic mass shift caused by the heavy isotope label are attributed to adducts between peptides derived from the target protein and the ligand, thus, revealing information about the ligand binding site within the target protein. Unmodified peptides identify the target protein itself, which has been 'fished' from the protein mixture using the ligand as bait.[70]

Conclusions and Outlook

Mass spectrometry presents a versatile method, which allows screening for protein–ligand interactions from minute sample amounts within a short time. It can be envisioned that mass spectrometers providing ultra-high resolution and high mass measurement accuracies, such as FTICR or orbitrap instruments will be increasingly used in the drug discovery process. Another important point is that the structural diversity and large number of protein–protein interfaces offer an enormous amount of new targets for the pharmaceutical industry.[72] Therefore, inhibitors of protein–protein interactions represent an attractive new class of drug candidates, which underlines the need to refine existing or develop novel MS-based strategies for analyzing protein–protein interactions.

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Keywords: ESI–MS · H/D exchange · MALDI–MS · noncovalent complexes · photoaffinity labeling

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