Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 528–538
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Review

Applications of mass spectrometry in early stages of target based drug discovery

Gejing Deng *, Gautam Sanyal

Department of Biochemistry, Infection Drug Discovery, AstraZeneca R&D Boston, 35 Gatehouse Drive, Waltham, MA 02451, USA

Received 5 July 2005; received in revised form 30 August 2005; accepted 30 August 2005
Available online 26 October 2005

Abstract

Mass spectrometry (MS) has been applied to drug discovery for many years. With the advent of new ionization techniques, MS has emerged as an important analytical tool in identification and characterization of protein targets, structure elucidation of synthetic compounds, and early drug metabolism and pharmacokinetics studies. Two MS-based strategies, function-based and affinity-based, have been employed in recent years for screening and evaluation of compounds. In the function-based approach, the effects of compounds on the biological activity of a target molecule are measured. In the affinity-based approach, compounds are screened based on their binding affinities to target molecules. The interaction between targets and compounds can be directly evaluated by monitoring the formation of non-covalent target–ligand complexes (direct detection) or indirectly evaluated by detecting the compounds after separating bound compounds from unbound (indirect detection). Various techniques including high performance liquid chromatography (HPLC)–MS, size exclusion chromatography (SEC)–MS, frontal affinity chromatography (FAC)–MS and desorption/ionization on silicon (DIOS)–MS can be applied. The recent advances, relative advantages, and limitations of each MS-based method as a tool in compound screening and compound evaluation in the early stages of drug discovery are discussed in this review.

Keywords: Mass spectrometry; Target based drug discovery; Function-based screening; Affinity-based screening

Contents

1. Introduction

Enzymes and receptors represent the most common drug targets identified to date. Target based drug discovery is an important strategy for developing new therapeutic agents. Essential activities in the early phases of execution of this strategy typically comprise identification and validation of a biological target, development of assays to identify compounds (hits) with activity against target function, and optimization of these hits using medicinal chemistry tools. The hit identification (HI) phase includes screening of the target against a compound library using a primary assay, followed by a thorough evaluation of the hits that emerge from the primary screening employing multiple approaches. Two general assay strategies for screening and hit evaluation (HE) are typically used. One comprises the use of an assay (or a set of assays), which monitors the modulation of the biological activity of the target by compounds, e.g. the catalytic activity of an enzyme. We will refer to this approach as "function-based screening". The second strategy, which uses the binding affinities of compounds for targets

* Corresponding author. Tel.: +1 781 839 4576; fax: +1 781 839 4600.
E-mail address: gejing.deng@astrazeneca.com (G. Deng).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.08.038

to screen for potential hits, is referred to as "affinity-based screening".

Mass spectrometry (MS) has been used in drug discovery for decades. With the advent of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) techniques, the use of MS as an analytical tool has extended to all stages of drug discovery, including target identification and characterization, structure elucidation of synthetic compounds and early drug metabolism and pharmacokinetics. Furthermore, in recent years, the potential of MS in compound screening and HE has been explored. MS has certain distinct advantages over conventional methods in function-based and affinity-based screening. Perhaps, the most attractive advantage of MS over other analytical techniques is its generic nature of detection based solely on the mass-to-charge ratio of a molecule. This is measured with exquisite accuracy and high sensitivity in modern day mass spectrometers, thereby providing an interference-free true fingerprint of the molecule.

2. Function-based screening

A protein target, such as an enzyme with a well-defined biological function, can be screened using functional assays to search for compounds that would modulate its function. The feasibility and success of such screening depend on development of assay methodologies that allow rapid and quantitative measurement of biological activity of macromolecular targets, e.g. by monitoring the time-dependent increase in the concentration of a reaction product or the concomitant disappearance of a substrate for an enzyme target. Compounds that specifically modulate the function of a target are often the starting molecular entities or "hits" from which "leads" and eventually "candidate drugs" may be potentially developed. These functional assays for studying target-compound interactions are used throughout the entire process of lead generation.

Among many methods applied for drug screening and HE, spectrophotometric or spectroflurometric assays have probably been the most commonly used. These types of assays are usually fast, relatively simple to use and amenable to automation. However, optical detection methods require that the analyte to be quantified has an intrinsic chromophore or fluorophore, or is readily convertible to a compound exhibiting well-resolved absorbance or fluorescence of sufficiently high signal at a desirable wavelength. An enzymatic coupling reaction often has to be introduced for such a conversion. In this case, the inhibition data need to be deconvoluted to ascertain that inhibition of target enzyme rather than the coupling enzyme(s) is being detected. The major drawback of these assays is that a high background may be produced due to optical interference from reaction substrates, screening compounds and/or other components required for enzyme activity. Other commonly used assays are radioactivity-based assays. These assays are very sensitive and can be formatted to high throughput automation, but they require radiolabeled substrates to be prepared and appropriate safety measures must be in place, including safe handling of hazardous radioactive waste.

Within the past decade, MS has become a powerful tool for studying enzyme kinetics and mode of inhibition as it can detect a wide range of molecules with high sensitivity and molecular selectivity. It has been widely applied in steady state enzyme kinetics to determine Michaelis–Menten constants (K_M) and turnover numbers (k_{cat}) [1–6] and in pre-steady state kinetics where transient enzyme reaction intermediates can be monitored, yielding information about individual steps along the catalytic pathway [7,8]. Another noteworthy advantage of MS over many other techniques is that it can be used to monitor and quantify multiple components simultaneously. Pi and Leary have developed a multiplex assay to study enzyme/substrate specificity, from which multiple substrates can be evaluated simultaneously in one assay [9]. As the MS technique detects native substrates or products directly and quantitatively, the catalytic mechanism of an enzyme can be studied conveniently [10,11]. The use of chromophores, radioactive labels or secondary enzymatic reactions irrelevant to the target enzyme reaction can be eliminated. Method development for an MS-based assay is usually rapid (typically 1–2 days) with appropriate instrumentation. False positives and false negatives are rare as the mass of an analyte or its fragments, generated by collision-induced fragmentation (CID) in a tandem mass spectrometer, is generally unique. A direct comparison between a spectrophotometric assay and an LC–MS assay for the same enzymatic reaction showed that the latter has a better sensitivity, lower background, lower limit of quantitation, wider dynamic range and better reproducibility [12].

For reactions in which target enzymes can function properly in simple reaction matrices, such as low concentrations of volatile buffer and salt, samples can be directly injected into a mass spectrometer without any column separation [1,9–11]. However, for reactions involving high concentrations of salts and buffer, a fast LC separation before MS detection is highly recommended to reduce ion suppression, which is a major cause of sensitivity loss. A divert valve is used to direct the salts eluted at the column front into waste to avoid contamination of the MS ion source. For a reaction conducted in a complex reaction matrix, a triple quadrupole mass spectrometer used at multiple reaction monitoring (MRM) mode is recommended to decrease possible chemical noise arising from the reaction matrix.

A common procedure applied in target-based functional screening is illustrated in Fig. 1. An enzyme reaction is performed in a 96- or 384-well plate. After a defined reaction time, the reaction is stopped by addition of a chemical, which can inactivate the enzyme. Most enzymes can be inactivated by changing the solution pH or adding an organic solvent. Alternatively, a metal chelator, such as EDTA can be used if a divalent cation, such as Mg(II) is required for enzyme activity. The quenched reaction mixture is then injected into an LC–MS system and the reaction product is quantified by MS. These assays can be used to screen a compound library at a defined concentration of each compound to obtain the percentage of inhibition (%I) or at a series of compound concentrations to obtain inhibition potency of a compound (IC_{50}).

LC–MS assays usually have a lower throughput compared to spectrophotometric or spectrofluorometric assays in which 96

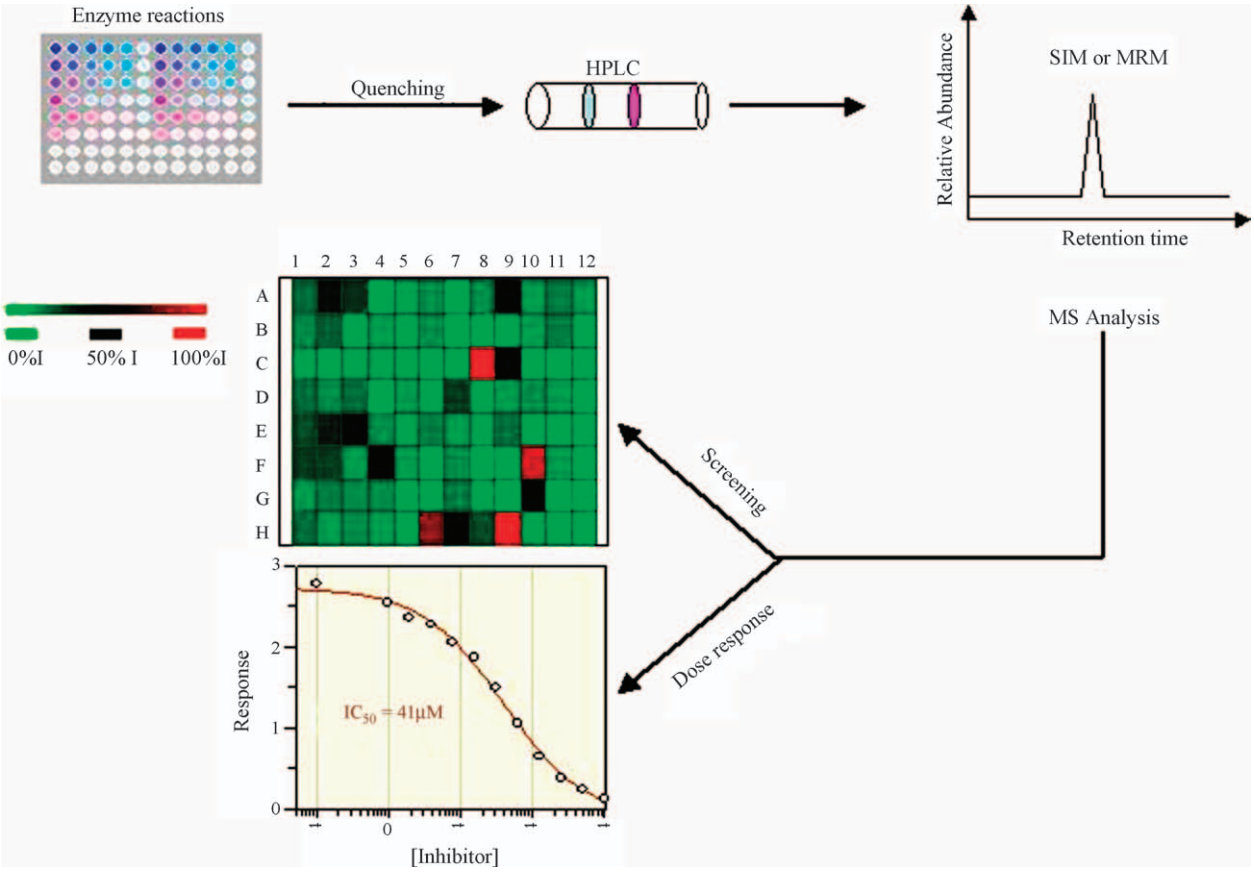

Fig. 1. A schematic representation of an LC–MS based enzyme inhibition assay. Analytes can be quantified by selected ion monitoring (SIM) or multiple reaction monitoring (MRM). Inhibition of a target enzyme can be evaluated at a single compound concentration to obtain %Inhibition. Heat map can be used to locate and compare potency of compounds in a 96-well format. Compounds can be also evaluated at serial concentrations to obtain IC_{50} (inhibitor concentration required for 50% inhibition).

or 384 data points can be obtained simultaneously by a plate reader. The reduction in throughput happens at the front-end, as the time spent in the LC step is rate limiting, although a short LC gradient is usually sufficient because MS provides another dimension of resolution. In addition to the time consumed in the gradient, LC columns require a certain amount of time to be reconditioned and re-equilibrated after each analysis. To address this issue, Agilent Technologies Inc. introduced an alternating column regeneration strategy (Fig. 2) in which two identical columns are switched between an eluent pump and a regeneration pump using a 2-position/10-port valve. While one column is performing separation and analysis, the other column is being regenerated and equilibrated. With an autosampler of the Agilent 1100 Series, it is also possible to perform overlapping injections. This means that while one sample is being analyzed, the next sample can be drawn into the sample loop and held there until the next injection. With this system, about 40% increase in throughput can be achieved. This system is inexpensive, simple to use and easy to maintain. To further increase the throughput of LC–MS assays, multiple high performance liquid chromatography (HPLC) coupled to a mass spectrometer can be applied [13]. Multiple flows from the respective columns can be introduced into a mass spectrometer interface sequentially or simultaneously. In the first method, samples are injected onto their respective columns in a staggered fashion. Each column utilizes its own set of pumps for mobile phase delivery. A valve selector is used to sequentially introduce the fraction of interest from each HPLC separation into the ionization source (Fig. 3A). In the second method, only one set of pumps is used to produce the main flow, which is then split into multiple sub-flows for individual columns. A four- or eight-channel multiplexed electrospray (MUX) ionization source (Waters Inc.) utilizes an indexed sampling rotor to permit one spray at a time to be introduced into the sampling cone of a mass spectrometer. Although fewer data points are collected during peak elution, resulting in decreased sensitivity, the technique allows analytes from multiple columns to be analyzed simultaneously (Fig. 3B) [13].

Desorption/ionization on silicon (DIOS) time-of-flight (TOF) is a matrix-free technique, where analyte molecules are trapped within a porous silicon surface from which they are laser-desorbed and ionized [14–16]. Unlike matrix-assisted laser desorption/ionization TOF–MS, the absence of matrix in DIOS allows analysis of small molecules below m/z 300. Therefore, this technique is an excellent tool for small molecule analysis. DIOS–MS without LC separation has been evaluated for studying enzyme kinetics and inhibition [14,17]. It has been shown that DIOS–TOF is as quantitative as LC–MS/MS,

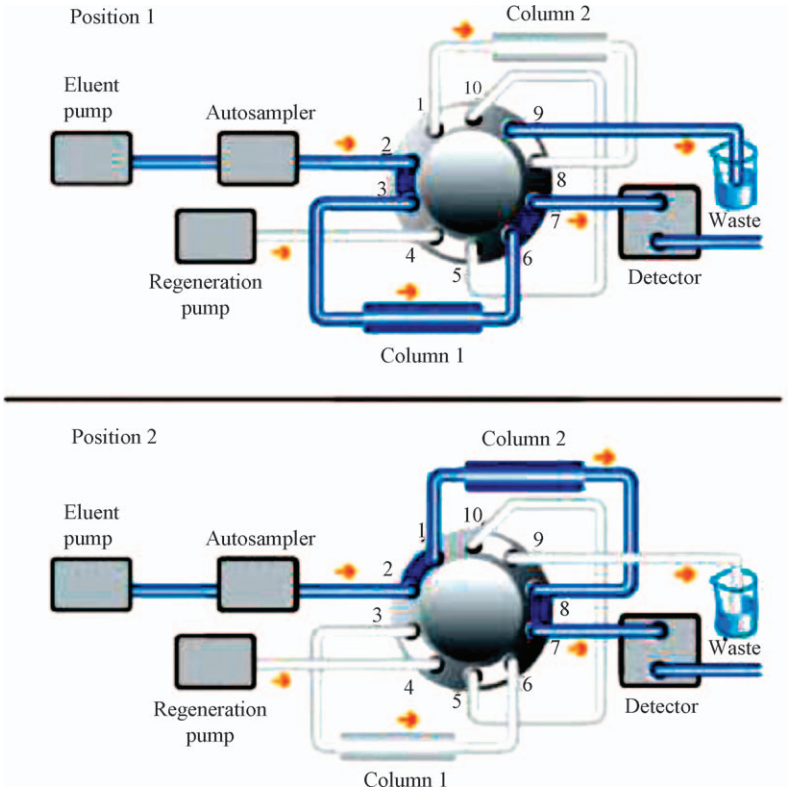

Fig. 2. Alternating column regeneration using a binary pump. In Position 1, Column 1 analyzes while Column 2 regenerates and flushes out to waste. In Position 2, Column 2 analyzes while Column 1 regenerates and flushes out to waste. Reproduced with permission from the web site of Agilent Technology Inc.

provided that an internal standard is used [17]. Very small amounts of sample, usually low picomoles are required for DIOS–TOF analysis. With a 200 Hz laser, a sampling rate of 1.6 s per sample can be achieved. In other words, a plate of 100 compounds can be screened in 160 s [18]. With the incorporation of electrospray sample deposition (ESD), more uniform deposition is achieved and this allows improved accuracy and reproducibility for quantitative analysis [18]. However, all studies reported so far involved enzyme reactions that can be carried out in very simple reaction matrices, such as a low concentration of volatile buffer (5–25 mM ammonium bicarbonate or ammonium citrate). In reality, many enzymes require cofactors, non-volatile salts and stabilizers to be fully functional. Without a separation step, or at least a desalting step, ion suppression by the reaction matrix could be severe, resulting in decreased sensitivity and reliability of quantitation.

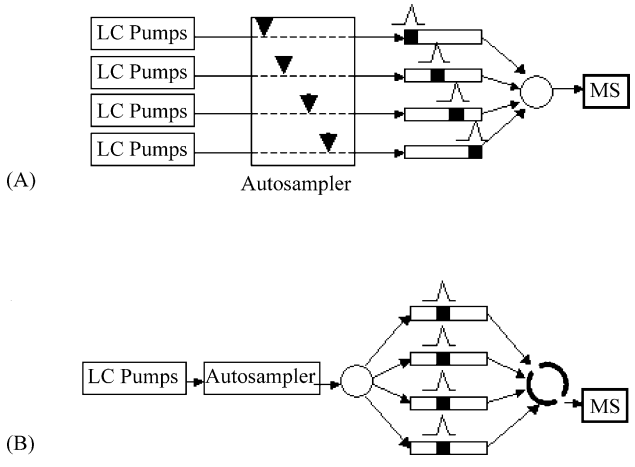

Fig. 3. Schematic representation of a parallel HPLC column configuration. (A) Each column uses its own set of LC pumps for mobile phase delivery. Staggered injection allows each analyte eluted at the defined retention time window to be analyzed sequentially by a single sprayer. (B) Parallel injection and separation. Analyte from each column is analyzed with indexed multiplex sprayer (MUX).

Currently, LC–MS is mostly used as an important tool in HE, after primary screening of a large compound library against a target using a non-MS-based high throughput assay. The number of hits generated by the primary screening is much smaller than those in the original library and is usually readily amenable to MS analysis in terms of throughput. The MS assay applied at this stage provides an independent and orthogonal confirmation of the validity of the hits offered by the primary assay. For reasons such as specificity and selectivity described earlier MS provides a powerful assay platform for HE and aids the process of moving forward with high quality hits that have the potential to become or be transformed to lead compounds.

With the introduction of more and more high throughput platforms, such as “Lab-on-a-tape” (BioTrove Inc.), MS is becoming a suitable tool for primary screening of large compound collections. An integrated microfluidic system has been developed for direct quantitation of analytes in complex reaction mixtures [19]. This system is designed for solution phase biochemical assays. Large numbers of compounds can be screened for biological activity against an enzyme target at the throughput of 4–5 s per sample.

3. Affinity-based screenings

Another approach for target-based screening involves the determination of the relative affinities of compounds for target macromolecules. Compared to function-based screenings, which require characterization of macromolecular targets, identification of appropriate substrates, and development of robust assays for each target, affinity-based screenings do not require knowledge of structure or function of a target, and do not require the development of target-specific assays. The affinity-based screenings are, therefore, very suitable for the targets identified and validated by strategies such as gene disruption and siRNA from which the linkage between the targets and disease phenotype have been confirmed but function of the genes or the targets may still be unknown [20]. Another advantage of affinity-based screening is that as the assay formats for different targets are relatively invariant, the promiscuous, frequent hitters can often be identified. On the other hand, the drawback of this strategy is that it will identify compounds simply based on their binding affinities for a target irrespective of whether or not the biological function of the target is affected. A variety of front-end affinity selection techniques have been applied in conjunction with MS to identify potential ligands. The screening process is initiated by forming a macromolecular target–ligand complex under native conditions. The non-covalent complexes can then be characterized either by direct detection of the complex by MS or by detection of bound compound after the compound is dissociated from the complex or by detection of unbound compound after its interaction with the target.

3.1. Direct detection of non-covalent macromolecule–ligand complexes

Although mass spectrometry has been widely used to identify and analyze small molecules for decades, it only became possible to analyze macromolecules such as proteins and nucleic acids when two soft ionization techniques, MALDI and electrospray ionization (ESI), were introduced in the late 1980s. ESI is capable of desorbing many non-covalent complexes, at close to their native solution state, into gas phase as charged ions. Samples are usually introduced to MS by direct infusion and the molecular weights of non-covalent complexes are measured directly. A mass accuracy of better than 0.01% can be usually achieved for protein complexes with molecular masses of <35 kD [21]. This high mass accuracy is far superior to that of traditional native gel electrophoresis and size exclusion chromatography (SEC) methods. Therefore, ESI–MS is ideal for the determination of masses of non-covalent complexes from which the stoichiometry of these complexes can be accurately obtained. Compared to other biophysical techniques, such as NMR spectroscopy and analytical ultracentrifugation, the major advantages of using mass spectrometry for studying non-covalent complexes are its speed and sensitivity. Only nanomole to picomole amounts of material are required and each analysis can be conducted in a few minutes. ESI–MS has been applied to studying various non-covalent interactions, including monitoring the dynamics of enzyme-catalyzed reactions [22], enzyme-inhibitor complexes [23], quadruplex structure of DNA [24], DNA–protein complexes [25–29], RNA–protein complex [30–32], DNA–RNA interactions [33], drug–DNA complexes [34] and protein–protein interaction [35]. The use of ESI–MS to monitor macromolecular complexes, which are involved in the chaperonin-assisted protein folding cycle has been demonstrated recently [36]. This adds an important new dimension for the application of MS to basic biological sciences [37].

However, to ensure that the native state of a complex is retained during MS analysis, appropriate ESI source parameters and solution conditions must be used [38]. Experimental conditions need to be carefully optimized to achieve the right balance between keeping the target–ligand complex intact and the efficiency of MS analysis. For example, orifice potential must be sufficient to ionize a protein complex but not too high to disrupt the non-covalent interaction. Unfortunately, proteins often require non-volatile salts, co-factors, additives, surfactants and neutral pH to maintain their native conformation and biological activity. These conditions are not ideal and can be problematic for MS detection where low pH and the presence of some organic solvent are usually preferred for efficient ionization. As a result, this approach is usually limited to those targets whose native state can be preserved at a relatively acidic pH (e.g. pH 5.5–6.5) and in low concentrations of volatile salts and buffers (e.g. 10–50 mM ammonium acetate, Tris acetate or ammonium bicarbonate).

In order to identify specific binders, a screening method should be carefully validated. Good correlation should exist between K_d values measured in gas phase (by MS) and in solution phase (by other techniques). A ligand with known solution K_d can be used for method development. It has been noted that this approach is especially useful for RNA targets as MS friendly conditions, such as methanol, have been reported to stabilize the tertiary structure of RNA [39] and up to 50% of an organic solvent has been used [40]. A number of MS-based assays for RNA targets have been developed [41–43]. These assays are extremely sensitive and can detect RNA binders with K_d values ranging from nanomolar to millimolar. The binding stoichiometry and dissociation constants for binding of aminoglycoside antibiotics to ribosomal RNA have been determined using these assays [40]. MS-based assays have also been used in a relatively high throughput fashion for affinity-based screenings of compounds against a 27-mer nucleotide RNA, an essential components of 16S rRNA A-site, which is responsible for binding of tRNA in translation [44,45]. Furthermore, these assays have helped to generate lead compounds that target ribosomal RNA, using structure–activity relations developed on the basis of binding affinities of known antibiotics to rRNA [46–50]. The perspective of MS as a drug discovery platform against RNA targets has been comprehensively reviewed [51].

Native proteins and protein–ligand complexes have far fewer charges than denatured proteins and, therefore, can be only detected at high values of m/z. Mass spectrometers capable of high mass measurements, such as quadrupole time-of-flight

(QTOF) instruments, are essential for studying non-covalent complexes. Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers have also been used for studying non-covalent complexes of smaller protein targets and ligands. Benner and coworkers have applied FT-ICR to identify and screen a 324-member peptide combinatorial library in a single experiment [52]. Bound ligands can be unambiguously identified following infrared multiphoton dissociation of non-covalent complexes. Using a similar approach, the binding affinities of more than 250 compounds can be ranked simultaneously [53]. The promise, pitfalls and prognosis of using MS to study non-covalent complexes have been reviewed extensively [54–57].

A lot of effort has been made to improve the throughput of affinity-based screening. A chip-based nanoelectrospray system, NanoMate, introduced by Advion BioSciences, allows samples to be introduced into a mass spectrometer in a fully automated, reproducible and robust manner. Each sample is processed using a separate tip and nozzle, achieving zero carry-over between samples. Using this system, a reproducible screening can be performed at a speed up to 50 times faster than NMR [58,59].

3.2. Indirect detection of non-covalent macromolecule–ligand complexes

The interaction between macromolecular targets and screening compounds can be indirectly evaluated by detecting the compounds, after separating bound and unbound compounds. A number of techniques including SEC [60–63] and molecular weight cut-off membranes [64–66] have been applied to separate the non-covalent complexes from unbound compounds. Determination of bound ligands requires the dissociation of the complex. Non-covalent complexes can be disrupted by denaturing conditions, such as 3% acetic acid in acetonitrile–water, and subjected to MS analysis by direct infusion [61]. The complexes can be also directly injected onto a reversed phase HPLC (RP-HPLC) column where the dissociation of the complexes is accomplished by raising the column temperature to, e.g. 60 °C, and by mobile phases used for RP-HPLC (usually acidic solutions, e.g. pH 2, containing organic solvent). Binding affinities can be measured at a constant protein concentration and varied concentrations of a compound or vice versa. A Scatchard plot or, for more accurate results, non-linear fit of a binding curve can be used to obtain the K_d value. One should keep in mind that the equilibrium between target macromolecule, compound and non-covalent complex is disturbed to some extent during size exclusion or ultrafiltration procedure. Therefore, binding constants determined by this method are usually somewhat higher than those obtained by equilibrium methods, such as isothermal titration calorimetry (ITC) and equilibrium dialysis. For example, the K_d value determined by SEC spin column separation followed by reversed phase LC–MS for warfarin binding to human serum albumin is about four times higher than that determined by ITC (39 μM by MS versus 10 μM by ITC; Deng, G. et al. unpublished data). To minimize the perturbation of equilibrium during SEC, the chromatographic separation should be carried out fast (e.g. ≤1 min) and at a relatively low temperature (e.g. 4 °C). It should also be noted that due to the perturbation of equilibrium during complex separation, this approach is not typically used to obtain binding stoichiometry. Competitive ligand displacement experiments against a known specific ligand can be carried out to identify specific binders and to determine K_d. The binding site can be further confirmed if site-specific mutants of the target molecule are available.

In an alternative method, the unbound or free compound is quantified by LC–MS after its separation from the macromolecular complex by SEC. [67]. The unbound compound retained by SEC column are recovered and analyzed by MS. An excess of target is typically used. The absence of a compound in the SEC retained fraction indicates strong affinity of the compound towards the target, while detection of a significant amount of a compound indicates weak or no binding of the compound. This method, though effective in finding strong binders, requires the recovery of unbound compounds from SEC, which usually involves serial washing steps, an elution step, and a subsequent sample-concentrating step before the sample can be analyzed by LC–MS.

To improve throughput, 96-well plates filled with preswollen Sephadex G-25 beads (0.35 mL per well, BHK Laboratories Inc.) and a 96-well plate bench-top centrifuge (Eppendorf) have been used for fast, parallel separation of the high MW non-covalent complex from unbound low MW molecules [67]. A similar method has been validated and developed into a lead discovery platform (SpeedScreen) for selection of high-affinity binders in an industrial screening environment [68]. Up to 600 compounds per well at 7 μM concentration each compound were incubated with 10 μM target in a 96-well pinhole plate. The samples were rapidly centrifuged through the pinhole into an SEC plate. The protein-bound ligands were then passed into the collection plate for subsequent LC–MS analysis. All sample handling steps and the analytics are rapid, robust, and largely automated. Another high throughput technology platform, termed ALIS™, has been introduced by NeoGenesis Pharmaceuticals Inc. ALIS™ is an integrated inline system comprising incubation of the compound mixture, separation of binders from non-binders by parallel SEC columns, separation of binders from protein by reversed phase chromatography, and ESI–MS. Up to 300,000 compounds per day per screening line can be screened [69]. High throughput ultrafiltration-based affinity screening has been also described [70].

Another procedure for detecting low molecular mass compounds non-covalently bound to macromolecular targets involves the use of an immobilized target. Three methods can be used. In the first method, MS is coupled to frontal affinity chromatography (FAC). A column is prepared containing a fixed amount of immobilized biological receptor or enzyme. A sample containing potential ligands or inhibitors is continuously infused through the column. The order of compound elution parallels their affinities for immobilized receptor or enzyme (Fig. 4A). The tightest binding ligand elutes last while compounds, which do not interact with the target elute at the void volume of the column. A target can be directly immobilized onto a stationary phase through reductive amination between the protein's primary amines and the aldehyde functional groups on beads, such as Aminilink coupling gel (Pierce).

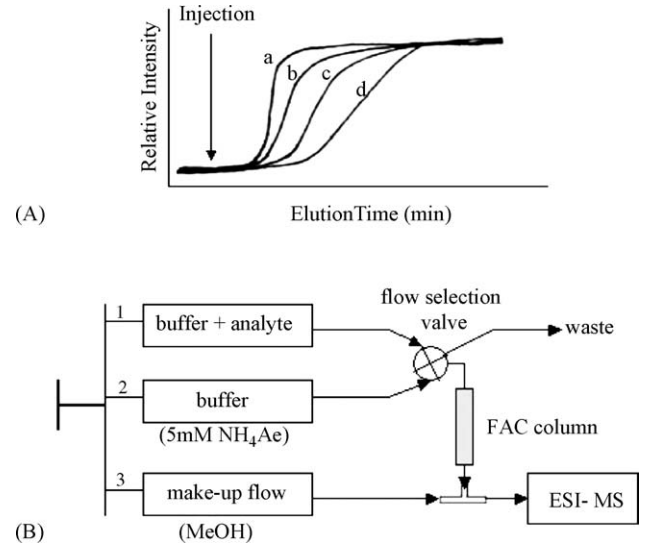

Fig. 4. Schematic diagrams of extracted ion chromatograms of compound a, b, c and d monitored by FAC–MS (A) and instrument configuration (B). FAC column is firstly equilibrated with buffer from syringe 2. The flow selection valve is then switched to syringe 1 (injection) to collect ion chromatogram.

Alternatively, the target can be indirectly immobilized onto the stationary phase via avidin. The latter method requires that the target molecule is pre-biotinylated before it is immobilized onto avidin beads. The apparatus used for FAC–MS analysis contains a multisyringe pump, which delivers three solutions in parallel, a switching valve, and a mass spectrometer (Fig. 4B). Two of the three syringes and a switching valve are used to deliver either the elution buffer or the compound solution into the mass spectrometer. The third syringe is used to deliver a make-up solution, through a post FAC column tee, which facilitates MS detection of compounds eluted from the column. The dissociation constants (K_d) of individual ligands in the mixture can be determined based on the FAC theory [71]. A void volume marker, a compound with no affinity for the immobilized target, is required to determine the column capacity, which is used to calculate the K_d value of an unknown ligand. By using an "indicator", a compound known to bind to a specific site on a protein target with a known affinity, such as a substrate of a target enzyme, competitive inhibitors in a compound mixture can be readily identified by the shifted elution volume of the indicator [72]. Other applications of using an indicator include: (1) determining if an individual ligand has a higher or a lower K_d value than the indicator, based on the shape of FAC–MS elution profile (for example, a "roll up" elution profile of a compound usually indicates that the compound binds to the target at lower affinity than the indicator [73,79]); (2) indirectly screening compounds that do not "fly" in the mass spectrometer and (3) screening multiple binding site binders simultaneously using multiple indicators. Slon-Usakiewicz et al. have recently demonstrated that FAC–MS has the ability to distinguish between competitive ligands for ATP and for substrate sites of a protein kinase C independently in the same experiment [74].

To reduce the consumption of screening compounds, micro-scale columns with column volume of 20–40 μL are typically used for screening. This approach is capable of measuring K_d values from low nanomolar to high micromolar, using sub-picomolar amounts of immobilized proteins. A number of drug targets including antibodies [75], lectins [76], growth factor receptor mimics [77] and enzymes [78,79] have been successfully used for ligand or inhibitor screening. FAC–MS can be also applied to differentiate the binding affinities of isomeric compounds without using an expensive separation process. A "multiple front" elution profile is usually observed if multiple isomeric compounds bind to a target with different affinities [79].

However, it should be noted that the FAC–MS approach is not applicable for ligands with very low on and off rates. K_d values measured by this approach may deviate from those measured in solution if a target protein cannot fully retain its native structure after immobilization. To minimize activity loss of a target, the number of sites on each target molecule used for immobilization should be controlled at a low level. The active site of a target should be protected during the immobilization process by using a known ligand, such as a substrate of an enzyme. K_d values of multiple compounds in a mixture can be estimated in a single experiment. However, the values obtained may be underestimated if several ligands in the mixture compete for the same binding site. It is suggested that for K_d measurement, each compound should be analyzed individually while for ranking purpose a group of compounds may be analyzed simultaneously. It has been shown that the ranking order of K_d values of compounds in a mixture is usually correct even though the K_d values deviate from those determined by other methods [78]. It has been reported that up to 200 compounds can be analyzed in a single experiment and 10,000 compounds can be screened per day per FAC–MS instrument (LeadFinder™ screening platform, Protana Inc.). A blank column containing no target protein should be prepared and used to evaluate non-specific binding of compounds to the stationary phase of the column and the biotin–avidin complex, if the latter were used for target immobilization. The advantages of this approach in screening combinatorial compound mixtures as well as the difficulties inherent in this screening method have been discussed [80].

FAC–MS can be also applied to evaluate the catalytic activity of an immobilized enzyme if the turn over of a substrate is monitored and quantified by MS. In these studies, reaction substrates along with a void volume marker of a fixed concentration are infused into a column for a defined time. The flow is then switched back to the elution buffer. The reaction product elutes from the column as a peak from which the amount of the product can be quantified. The void volume marker used here for measuring elution volumes of ligands can be also conveniently used as an internal standard. Kinetic constants and modes of inhibition can be studied if the flow rate and the reaction time are controlled under conditions of <10% of substrate conversion ensuring that the initial velocity of an enzyme reaction is measured [78].

The second method is to "fish" for interactions of interest with target molecules immobilized on a platform from which MS can be performed directly. Affinity DIOS–TOF MS has proven to be

very promising for screening small ligands. In this method, a target protein is immobilized on the porous silicon (pSi) probe. The probe is then incubated with a mixture of compounds before it is subjected to a washing step to remove unbound compounds. Subsequently, the probe is inserted into a mass spectrometer for analysis. The bound compound is desorbed and ionized directly from the immobilized probe and is analyzed by TOF–MS [81]. Zou et al. have demonstrated that bovine serum albumin (BSA), which was immobilized on pSi, could be used for identifying BSA binding ligands, such as ketoprofen. Sulpride, known to be a non-binder of BSA, was not detectable by affinity DIOS–MS [81]. A more delicate affinity DIOS system that contains a cleavable linker, which can be cleaved by the DIOS laser pulse, has been developed recently [82]. This system, combined with the tethering technology [83], has been demonstrated to be suitable for profiling enzyme active sites [84].

A number of on-chip affinity purification and mass detection of this type have also been described for MALDI–TOF MS [85–92]. However, the matrix used for desorption and ionization in MALDI usually generates strong matrix-related background which can obscure or even suppress signals from low-mass molecules. Affinity MALDI–TOF MS is therefore, generally, limited to identifying larger interacting partners of immobilized proteins, such as peptides and proteins. The examples include rapid identification of the antigenic determinant for an antibody using a monoclonal antibody immobilized to agarose beads after in situ proteolysis of the immobilized antigen–antibody complex followed by MALDI–TOF MS [91], and identification of components in urine which displayed the carbohydrate binding motif by lectin-based affinity capture and MALDI–MS analysis [92].

Similar to the method mentioned earlier by which unbound compounds are analyzed by LC–MS after its separation from the macromolecular complex by SEC [67], the third method involving immobilized target molecules is based on comparison of MS spectra before and after compounds interact with the immobilized macromolecular target. The compound mixture is firstly sprayed into a mass spectrometer, and spectrum showing the masses of all compounds is obtained. The mixture is subsequently incubated with target molecules immobilized on beads. The incubation mixture is then centrifuged. An aliquot of the supernatant is again analyzed by ESI–MS. Potential ligands are identified by comparison of the spectra before and after incubation with the immobilized enzyme. Inactive compounds show no change in ion intensity after incubation whereas active ligands exhibit a visible decrease in ion abundance or total disappearance from the spectrum. To ensure efficient ionization and detection of all compounds in a mixture, MS analysis should be performed in a positive/negative polarity switching mode. This approach has been applied to screen combinatorial libraries of up to 19 compounds in a single experiment [93]. Potential binders with K_d of approximately 100 μM or lower can be identified by this approach. Again, a molar excess of immobilized target molecule relative to the total molar concentration of compounds in a library should be used so that ample binding sites are available for ligand binding during incubation. The advantages of this method are that it does not require dissociation of the target–ligand complex and chromatography is not necessary. However, the procedure of recovering unbound ligands, which involves multiple washings and centrifugations, is tedious and time consuming. Similar to the FAC–MS method, non-specific binding occurring between the ligands, especially by hydrophobic compounds, and gel beads can occur [93] and should be evaluated by a control experiment where compounds are incubated with blank gel beads containing no immobilized target molecules. Strategies discussed above for FAC–MS approach, such as protection of active site of a target during immobilization and controlling the number of immobilization site on each target molecule should be as well applied here to reduce the loss of activity of the immobilized target.

4. Summary

Target based screening is one of the most important strategies in the early phase of a drug discovery effort to generate lead molecules. Mass spectrometry has become an important tool for target characterization, compound screening and hit evaluation. Two strategies have been discussed in this review. One measures the effects of compounds on the biological activity of a target molecule (function-based screening) and the other determines the binding affinities of compounds for target molecules (affinity-based screening). Methods that have been used for these two screening strategies are summarized in Table 1.

Both function and affinity-based screenings are important approaches in target based drug discovery. The two strategies complement each other. A lack of correlation between inhibitory activity and binding can be used as a diagnostic tool for compound deprioritization. To increase the level of confidence, key compounds identified by function-based screenings should be evaluated to ensure that the inhibitory activity seen is not due to inappropriate mechanisms, such as compound aggregation, interactions with substrates or detection reagents, and non-specific binding to or precipitation of the target of interest. On the other hand, compounds identified by affinity screening should ideally be evaluated by a function-based assay to ensure that the binding of the compounds to the target results in the modulation of the target function.

Although a lot of progress has been made in improving the throughput of mass spectrometry-based assays, they are still limited mostly to screenings of small compound libraries. They cannot yet routinely achieve the throughput of many commonly used high throughput screening assays, such as spectrophotometric, fluorometric and Scintillation Proximity Assays. However, they are especially useful as secondary assays in hit evaluation following a primary high throughput screening campaign. An orthogonal, secondary assay is critical to identification of false positives and confirmation of true hits. MS-based assays are also valuable for monitoring enzyme kinetics when no simple spectrophotometric assay is feasible or in situations where very high sensitivity and specificity are desired. With the introduction of more and more high throughput MS platforms, it is expected that mass spectrometry will play increasingly important roles in target-based screenings in early stages of drug discovery.

Table 1
MS-based screening methods

Analytes	Methods	Notes and applications
Function-based		
Reaction product(s) or substrate(s)	MS	For reactions conducted in complex matrix, LC–MS is recommended
	LC–MS	I% and IC_{50} measurements [12,17,18] Studying enzyme kinetics [1–8]
	DIOS–MS	High throughput platforms: MUX [13]; "Lab-on-a-tape" [19]; DIOS–MS plate reader [18]
Affinity-based		
Direct detection		
Non-covalent target/compound complex	MS by direct infusion	To obtain binding stoichiometry and K_d [36,48,52,53,42] Ensure to keep complex intact during analysis [40] High throughput platform: NanoMate [38,39]
Indirect detection		
Free targets		
Bound compounds	SEC or ultrafiltration → inject non-covalent complex to (RP-HPLC)–MS	Ligand screening and K_d estimation [60–66,68] Fast SEC/ultrafiltration separation required Dissociate non-covalent complex by PR-HPLC or by, e.g. 3% HAc in 50/50 ACN/H_2O High throughput platforms: SpeedScreen [68]; ALIS^{TM} [69]
Unbound compounds	SEC → recovers from SEC → MS	Ligand screening [67] Ensure excess target used Binders show decreased MS intensity or not show at all
Immobilized targets		
All compounds	FAC–MS	K_d measurement [71] Screening ligands and ranking ligand binding orders [75–79] Use indicators to study specific bindings and to screen compds. that do not "fly" in MS [72–74] Study binding affinities of isomeric compds [79]
Bound compound	Affinity DIOS-MS Affinity MALDI-MS	Target directly immobilized on DIOS or MALDI probe Ligand screening [81] Affinity MALDI–MS is used to identify larger binders [91,92]
Unbound compound	Centrifugation → MS	Binders identified based on MS spectra obtained before and after compounds interact with target immobilized on beads [93] Ensure excess target used

References

[1] N. Pi, J.I. Armstrong, C.R. Bertozzi, J.A. Leary, Biochemistry 41 (2002) 13283–13288.
[2] X. Ge, T.L. Sirich, M.K. Beyer, H. Desaire, J.A. Leary, Anal. Chem. 73 (2001) 5078–5082.
[3] A.J. Norris, J.P. Whitelegge, K.F. Faull, T. Toyokuni, Biochemistry 40 (2001) 3774–3779.
[4] B. Bothner, R. Chavez, J. Wei, C. Strupp, Q. Phung, A. Schneemann, G. Siuzdak, J. Biol. Chem. 275 (2000) 13455–13459.
[5] F.Y. Hsieh, X. Tong, T. Wachs, B. Ganem, J. Henion, Anal. Biochem. 229 (1995) 20–25.
[6] R.P. Newton, A.M. Evans, J.I. Langridge, T.J. Walton, F.M. Harris, A.G. Brenton, Anal. Biochem. 224 (1995) 32–38.
[7] C.T. Houston, W.P. Taylor, T.S. Widlanski, J.P. Reilly, Anal. Chem. 72 (2000) 3311–3319.
[8] D.L. Zechel, L. Konermann, S.G. Withers, D.J. Douglas, Biochemistry 37 (1998) 7664–7669.
[9] N. Pi, J.A. Leary, J. Am. Soc. Mass Spectrom. 15 (2004) 233–243.
[10] N. Pi, M.B. Hoang, H. Gao, J.D. Mougous, C.R. Bertozzi, J.A. Leary, Anal. Biochem. 341 (2005) 94–104.
[11] N. Pi, Y. Yu, J.D. Mougous, J.A. Leary, Protein Sci. 13 (2004) 903–912.
[12] G. Deng, R.F. Gu, S. Marmor, S.L. Fisher, H. Jahic, G. Sanyal, J. Pharm. Biomed. Anal. 35 (2004) 817–828.
[13] G. Hopfgartner, E. Bourgogne, Mass Spectrom. Rev. 22 (2003) 195–214.
[14] J.J. Thomas, Z. Shen, J.E. Crowell, M.G. Finn, G. Siuzdak, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 4932–4937.
[15] J. Wei, J.M. Buriak, G. Siuzdak, Nature 399 (1999) 243–246.
[16] E.P. Go, J.E. Prenni, J. Wei, A. Jones, S.C. Hall, H.E. Witkowska, Z. Shen, G. Siuzdak, Anal. Chem. 75 (2003) 2504–2506.
[17] D.B. Wall, J.W. Finch, S.A. Cohen, Rapid Commun. Mass Spectrom. 18 (2004) 1482–1486.
[18] Z. Shen, E.P. Go, A. Gamez, J.V. Apon, V. Fokin, M. Greig, M. Ventura, J.E. Crowell, O. Blixt, J.C. Paulson, R.C. Stevens, M.G. Finn, G. Siuzdak, Chembiochem. 5 (2004) 921–927.
[19] C.C. Ozbal, W.A. LaMarr, J.R. Linton, D.F. Green, A. Katz, T.B. Morrison, C.J. Brenan, Assay Drug Dev. Technol. 2 (2004) 373–381.

[20] K.M. Comess, M.E. Schurdak, Curr. Opin. Drug Discov. Devel. 7 (2004) 411–416.
[21] B.N. Pramanik, P.L. Bartner, U.A. Mirza, Y.H. Liu, A.K. Ganguly, J. Mass Spectrom. 33 (1998) 911–920.
[22] M. Ishigai, J.I. Langridge, R.S. Bordoli, Anal. Biochem. 298 (2001) 83–92.
[23] C. Darmanin, G. Chevreux, N. Potier, A. Van Dorsselaer, I. Hazemann, A. Podjarny, O. El-Kabbani, Bioorg. Med. Chem. 12 (2004) 3797–3806.
[24] D.R. Goodlett, D.G. Camp 2nd, C.C. Hardin, M. Corregan, R.D. Smith, Biol. Mass Spectrom. 22 (1993) 181–183.
[25] S. Akashi, R. Osawa, Y. Nishimura, J. Am. Soc. Mass Spectrom. 16 (2005) 116–125.
[26] A. Kapur, J.L. Beck, S.E. Brown, N.E. Dixon, M.M. Sheil, Protein Sci. 11 (2002) 147–157.
[27] N. Potier, L.J. Donald, I. Chernushevich, A. Ayed, W. Ens, C.H. Arrowsmith, K.G. Standing, H.W. Duckworth, Protein Sci. 7 (1998) 1388–1395.
[28] X. Cheng, P.E. Morin, A.C. Harms, J.E. Bruce, Y. Ben-David, R.D. Smith, Anal. Biochem. 239 (1996) 35–40.
[29] M. Przybylski, J. Kast, M.O. Glocker, E. Durr, H.R. Bosshard, S. Nock, M. Sprinzl, Toxicol. Lett. 82–83 (1995) 567–575.
[30] N. Hagan, D. Fabris, Biochemistry 42 (2003) 10736–10745.
[31] K.A. Sannes-Lowery, P. Hu, D.P. Mack, H.Y. Mei, J.A. Loo, Anal. Chem. 69 (1997) 5130–5135.
[32] H.Y. Mei, D.P. Mack, A.A. Galan, N.S. Halim, A. Heldsinger, J.A. Loo, D.W. Moreland, K.A. Sannes-Lowery, L. Sharmeen, H.N. Truong, A.W. Czarnik, Bioorg. Med. Chem. 5 (1997) 1173–1184.
[33] S.A. Hofstadler, R.H. Griffey, Chem. Rev. 101 (2001) 377–390.
[34] A. Kapur, J.L. Beck, M.M. Sheil, Rapid Commun. Mass Spectrom. 13 (1999) 2489–2497.
[35] J.A. Loo, T.P. Holler, S.K. Foltin, P. McConnell, C.A. Banotai, N.M. Horne, W.T. Mueller, T.I. Stevenson, D.P. Mack, Proteins. 2 (Suppl.) (1998) 28–37.
[36] E. van Duijn, P.J. Bakkes, R.M. Heeren, R.H. van den Heuvel, H. van Heerikhuizen, S.M. van der Vies, A.J. Heck, Nat. Methods 2 (2005) 371–376.
[37] C.V. Robinson, Nat. Methods 2 (2005) 331–332.
[38] K. Benkestock, G. Sundqvist, P.O. Edlund, J. Roeraade, J. Mass Spectrom. 39 (2004) 1059–1067.
[39] R. Shiman, D.E. Draper, J. Mol. Biol. 302 (2000) 79–91.
[40] K.A. Sannes-Lowery, R.H. Griffey, S.A. Hofstadler, Anal. Biochem. 280 (2000) 264–271.
[41] R.H. Griffey, S.A. Hofstadler, K.A. Sannes-Lowery, D.J. Ecker, S.T. Crooke, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 10129–10133.
[42] R.H. Griffey, K.A. Sannes-Lowery, J.J. Drader, V. Mohan, E.E. Swayze, S.A. Hofstadler, J. Am. Chem. Soc. 122 (2000) 9933–9938.
[43] K.A. Sannes-Lowery, J.J. Drader, R.H. Griffey, S.A. Hofstadler, Trends Anal. Chem. 19 (2000) 481–491.
[44] S.A. Hofstadler, K.A. Sannes-Lowery, S.T. Crooke, D.J. Ecker, H. Sasmor, S. Manalili, R.H. Griffey, Anal. Chem. 71 (1999) 3436–3440.
[45] L.L. Cummins, S. Chen, L.B. Blyn, K.A. Sannes-Lowery, J.J. Drader, R.H. Griffey, S.A. Hofstadler, J. Nat. Prod. 66 (2003) 1186–1190.
[46] Y. Ding, S.A. Hofstadler, E.E. Swayze, L. Risen, R.H. Griffey, Angew. Chem. Int. Ed. 42 (2003) 3409–3412.
[47] B. Wu, J. Yang, D. Robinson, S. Hofstadler, R. Griffey, E.E. Swayze, Y. He, Bioorg. Med. Chem. Lett. 13 (2003) 3915–3918.
[48] Y. He, J. Yang, B. Wu, D. Robinson, K. Sprankle, P.P. Kung, K. Lowery, V. Mohan, S. Hofstadler, E.E. Swayze, R. Griffey, Bioorg. Med. Chem. Lett. 14 (2004) 695–699.
[49] E.E. Swayze, E.A. Jefferson, K.A. Sannes-Lowery, L.B. Blyn, L.M. Risen, S. Arakawa, S.A. Osgood, S.A. Hofstadler, R.H. Griffey, J. Med. Chem. 45 (2002) 3816–3819.
[50] P.H. Seeberger, M. Baumann, G.T. Zhang, T. Kanemitsu, E.E. Swayze, S.A. Hofstadler, R.H. Griffey, Synlett. 9 (2003) 1323–1326 (special issue).
[51] S.A. Hofstadler, R.H. Griffey, Curr. Opin. Drug Discov. Devel. 3 (2000) 423–431.
[52] M. Wigger, J.R. Eyler, S.A. Benner, W. Li, A.G. Marshall, J. Am. Soc. Mass Spectrom. 13 (2002) 1162–1169.
[53] J. Gao, X. Cheng, R. Chen, G.B. Sigal, J.E. Bruce, B.L. Schwartz, S.A. Hofstadler, G.A. Anderson, R.D. Smith, G.M. Whitesides, J. Med. Chem. 39 (1996) 1949–1955.
[54] J.A. Loo, Mass Spectrom. Rev. 16 (1997) 1–23.
[55] M. Przybylski, M.O. Glocker, Angew. Chem. Int. Ed. 35 (1996) 807–826.
[56] D.L. Smith, Z.Q. Zhang, Mass Spectrom. Rev. 13 (1994) 411–429.
[57] R.D. Smith, K.J. Light-Wahl, Biol. Mass Spectrom. 22 (1993) 493–501.
[58] K. Benkestock, C.K. Van Pelt, T. Akerud, A. Sterling, P.O. Edlund, J. Roeraade, J. Biomol. Screen 8 (2003) 247–256.
[59] S. Zhang, C.K. Van Pelt, D.B. Wilson, Anal. Chem. 75 (2003) 3010–3018.
[60] Y.M. Dunayevskiy, J.J. Lai, C. Quinn, F. Talley, P. Vouros, Rapid Commun. Mass Spectrom. 11 (1997) 1178–1184.
[61] M.M. Siegel, K. Tabei, G.A. Bebernitz, E.Z. Baum, J. Mass Spectrom. 33 (1998) 264–273.
[62] R.G. Davis, R.J. Anderegg, S.G. Blanchard, Tetrahedron 55 (1999) 11653–11667.
[63] K.F. Blom, B.S. Larsen, C.N. McEwen, J. Comb. Chem. 1 (1999) 82–90.
[64] Y.Z. Zhao, R.B. van Breemen, D. Nikolic, C.R. Huang, C.P. Woodbury, A. Schilling, D.L. Venton, J. Med. Chem. 40 (1997) 4006–4012.
[65] R.B. van Breemen, C.R. Huang, D. Nikolic, C.P. Woodbury, Y.Z. Zhao, D.L. Venton, Anal. Chem. 69 (1997) 2159–2164.
[66] R. Wieboldt, J. Zweigenbaum, J. Henion, Anal. Chem. 69 (1997) 1683–1691.
[67] P.A. Wabnitz, J.A. Loo, Rapid Commun. Mass Spectrom. 16 (2002) 85–91.
[68] I. Muckenschnabel, R. Falchetto, L.M. Mayr, I. Filipuzzi, Anal. Biochem. 324 (2004) 241–249.
[69] M.H. Alaoui-lsmaili, P.T. Lomedico, S. Jindal, Drug Discov. Today 7 (2002) 292–294.
[70] J. Qian, M.J. Voorbach, J.R. Huth, M.L. Coen, H. Zhang, S.C. Ng, K.M. Comess, A.M. Petros, S.H. Rosenberg, U. Warrior, D.J. Burns, Anal. Biochem. 328 (2004) 131–138.
[71] K. Kasai, Y. Oda, N. Nishikata, S. Ishii, J. Chromat. Biomed. Appl. 376 (1986) 33–47.
[72] N.W. Chan, D.F. Lewis, P.J. Rosner, M.A. Kelly, D.C. Schriemer, Anal. Biochem. 319 (2003) 1–12.
[73] J.J. Slon-Usakiewicz, W. Ng, J.E. Foster, J.R. Dai, E. Deretey, L. Toledo-Sherman, P.R. Redden, A. Pasternak, N. Reid, J. Med. Chem. 47 (2004) 5094–5100.
[74] J.J. Slon-Usakiewicz, J.R. Dai, W. Ng, J.E. Foster, E. Deretey, L. Toledo-Sherman, P.R. Redden, A. Pasternak, N. Reid, Anal. Chem. 77 (2005) 1268–1274.
[75] D.C. Schriemer, D.R. Bundle, L. Li, O. Hindsgaul, Angew. Chem. Int. Ed. 37 (1998) 3383–3387.
[76] B. Zhang, M.M. Palcic, H. Mo, I.J. Goldstein, O. Hindsgaul, Glycobiology 11 (2001) 141–147.
[77] L. Zhu, L. Chen, H. Luo, X. Xu, Anal. Chem. 75 (2003) 6388–6393.
[78] B. Zhang, M.M. Palcic, D.C. Schriemer, G. Alvarez-Manilla, M. Pierce, O. Hindsgaul, Anal. Biochem. 299 (2001) 173–182.
[79] N.W. Chan, D.F. Lewis, S. Hewko, O. Hindsgaul, D.C. Schriemer, Comb. Chem. High Throughput Screen 5 (2002) 395–406.
[80] D.C. Schriemer, O. Hindsgaul, Comb. Chem. High Throughput Screen 1 (1998) 155–170.
[81] H. Zou, Q.-C. Zhang, Z. Guo, B. Guo, Q. Zhang, X. Chen, Angew. Chem. Int. Ed. 41 (2002) 646–648.
[82] J.C. Meng, C. Averbuj, W.G. Lewis, G. Siuzdak, M.G. Finn, Angew. Chem. Int. Ed. 43 (2004) 1255–1260.
[83] G.C. Adam, J. Burbaum, J.W. Kozarich, M.P. Patricelli, B.F. Cravatt, J. Am. Chem. Soc. 126 (2004) 1363–1368.
[84] J.C. Meng, G. Siuzdak, M.G. Finn, Chem. Commun. 18 (2004) 2108–2109.
[85] H. Neubert, E.S. Jacoby, S.S. Bansal, R.K. Iles, D.A. Cowan, A.T. Kicman, Anal. Chem. 74 (2002) 3677–3683.
[86] J. Su, M. Mrksich, Angew. Chem. Int. Ed. 41 (2002) 4715–4718.

[87] T. Natsume, H. Nakayama, T. Isobe, Trends Biotechnol. 19 (2001) S28–S33.
[88] J.O. Koopmann, J. Blackburn, Rapid Commun. Mass Spectrom. 17 (2003) 455–462.
[89] N. Tang, P. Tornatore, S.R. Weinberger, Mass Spectrom. Rev. 23 (2004) 34–44.
[90] L.W. Dick Jr., L.B. McGown, Anal. Chem. 76 (2004) 3037–3041.
[91] D.I. Papac, J. Hoyes, K.B. Tomer, Protein Sci. 3 (1994) 1485–1492.
[92] J. Bundy, C. Fenselau, Anal. Chem. 71 (1999) 1460–1463.
[93] M.T. Cancilla, M.D. Leavell, J. Chow, J.A. Leary, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 12008–12013.