# Applications of ESI-MS in drug discovery: interrogation of noncovalent complexes

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Abstract | For many years, analytical mass spectrometry has had numerous supporting roles in the drug development process, including the assessment of compound purity; quantitation of absorption, distribution, metabolism and excretion; and compound-specific pharmacokinetic analyses. More recently, mass spectrometry has emerged as an effective technique for identifying lead compounds on the basis of the characterization of noncovalent ligand–macromolecular target interactions. This approach offers several attractive properties for screening applications in drug discovery compared with other strategies, including the small quantities of target and ligands required, and the capacity to study ligands or targets without having to label them. Here, we review the application of electrospray ionization mass spectrometry to the interrogation of noncovalent complexes, highlighting examples from drug discovery efforts aimed at a range of target classes.

Mass spectrometry is increasingly perceived to be an essential tool in the drug discovery process at many (if not all) of the key steps in the development of novel therapeutics, including lead identification, assessment of compound purity, toxicology and pharmacokinetics, and quality control of bulk drug substance. Indeed, a number of recently published papers, books and special issues of topical journals have been devoted to describing the many diverse roles mass spectrometry has in the overall drug discovery and development process (for examples, see REFS 1–5). Improvements in mass spectrometry performance (in terms of sensitivity, resolution and mass accuracy), coupled with increasingly sophisticated sample purification and automation approaches, have opened the door for many novel analytical applications related to drug discovery and development. Many of these methods, which often couple high-performance liquid chromatography (HPLC) or tandem mass spectrometry (MS/MS) with electrospray ionization (ESI), are now well established and accepted in industrial and academic research and development laboratories around the world.

Less mature, but potentially even more valuable to the drug discovery community, are mass spectrometry-based methods for lead identification. In these methods the inherent multiplexing capability and automatability of the mass spectrometer can be exploited to rapidly screen drug candidates for specific interactions with targets of interest (FIG. 1; TABLE 1). These approaches can be separated into two primary categories: those that detect ligands following interaction with a target and allow inferences of affinity (and/or specificity) of interaction by the abundance of the 'free' ligand; and those that directly detect ligand–target noncovalent complexes in the gas phase and allow inferences of affinity (and/or specificity) of the ligand–target interaction by the abundance of the noncovalent complex. Both of these approaches are increasingly being utilized in the drug discovery process. In this review, we highlight recent examples from our laboratory and from the literature in which ESI-MS is used to directly characterize noncovalent complexes in support of drug discovery efforts against several classes of target.

## Interrogation of ligand–target complexes

Structured biomolecules remain the principal target for drug discovery as they are at the core of most essential biological processes. Protein function is dependent on molecular conformation, which in turn modulates intermolecular interactions. In drug development, ligands that bind specifically to targeted proteins either induce a conformational change or 'outcompete' the active site for a natural ligand, thereby interrupting key pathways associated with infection or disease. Similarly,

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RNA molecules are a crucial component of a number of core biological processes, including peptide synthesis, mRNA splicing, tRNA processing, and regulation of both transcription and translation. These processes can be targeted with small molecules that bind to structured regions of RNA to elicit a therapeutic effect. For example, aminoglycoside antibiotics inhibit bacterial growth by disrupting essential RNA–protein and RNA–RNA interactions[6,7]. Paromomycin, one of the most widely studied aminoglycosides, binds to the decoding region of the prokaryotic 16S rRNA (the A-site), with a dissociation constant ($K_d$) of <200 nM, and induces misreading of the genetic code during translation[8].

Ganem *et al.*[9] were the first to publish a paper demonstrating that molecules that were noncovalently associated in solution could be transferred into the gas phase with ESI and detected as an intact complex. Most importantly, in that initial study, the specificity of the interaction between FK-binding protein (molecular mass of 11,812 Da) and the ligand FK506 was maintained; compelling evidence was presented that the ESI-MS signals arose from the intact, specific, noncovalent complex, and not from nonspecific aggregation[9]. Although this initial report was met with some scepticism by the mass spectrometry community, the utility of ESI-MS for the ionization, detection and characterization of noncovalent complexes of nearly every type of biomolecule has now been described in ~300 publications and numerous review articles (for examples, see REFS 10–16). ESI-MS has been used to characterize various features of protein–protein, protein–DNA, protein–RNA and DNA–DNA complexes, including macromolecular- and ligand-binding stoichiometry and solution binding affinities[17]. Mass spectrometry has a major advantage over other biophysical tools — the identities of different complexes can be determined directly, as the mass of each molecule serves as the intrinsic detection 'label'. In addition to exploiting the '*x* axis' of the mass spectrum (that is, the mass-to-charge ratio, *m/z*), the '*y* axis' of the mass spectrum (that is, abundance/intensity) provides important information about affinity and specificity. The combined information from the *x* and *y* axes can be used to rapidly determine which compounds from a mixture bind to which targets, and with what relative affinity. Molecular interactions with dissociation constants ranging from nM to mM have been characterized using ESI-MS[18–20].

Although noncovalent complexes have been studied by many methods — including ultracentrifugation, surface plasmon resonance, calorimetry, fluorescence, circular dichroism, light scattering, infrared spectroscopy, Raman spectroscopy, electron spin spectroscopy, NMR and crystallography[21–23] — mass spectrometry has several potential advantages over these more conventional methods. First, mass spectrometry does not require that the ligands or targets be modified by labelling or binding to a surface. This is an advantage because such modifications can have deleterious effects on binding properties. Second, the sensitivity of mass spectrometry allows microgram quantities of target to be used instead of multi-milligram quantities typically required by NMR and crystallography-based methods. The use of small quantities of material is important as it is sometimes not technically feasible, or the cost is prohibitive, to generate large quantities of target for screening. Third, methods based on mass spectrometry are rapid and automatable. Each step, from sample introduction to mass analysis to spectral interpretation, can be fully automated to run around the clock — an important attribute required for screening increasingly large compound collections. Fourth, constraints on the purity of both targets and ligands are significantly
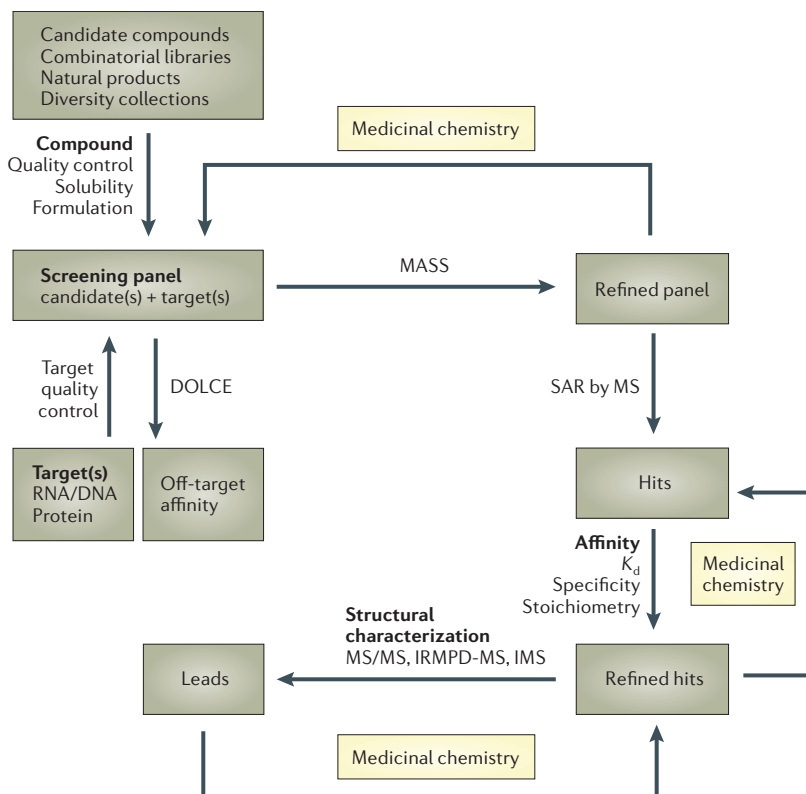


Figure 1 | **Potential applications of ESI-MS in drug discovery.** Candidate compounds, which might comprise combinatorial libraries, archived diversity libraries or natural product fractions, are first solubilized and possibly characterized by mass spectrometry for purity. Macromolecular drug targets such as structured RNA motifs, DNA constructs or protein targets could initially be analysed by mass spectrometry for purity or solubility. After mixing the solubilized compound collection and macromolecular target(s) in solution conditions that maintain the higher-order structure of the targets to produce a primary screening panel, affinity-based techniques such as multi-target affinity/specificity screening (MASS) can be used to select compounds with favourable binding to the target(s) of interest. Once initial binders are identified, the affinity and specificity can be optimized by multiple cycles of medicinal chemistry and re-screening to produce a set of high-affinity compounds. Structure–activity relationship determination by mass spectrometry (SAR by MS) can also be used to analyse small molecules for cooperative, concurrent or competitive binding; those molecules that bind the target concurrently or cooperatively can be synthetically linked into new molecular constructs with improved binding characteristics. In addition, compounds with unfavourable off-target binding characteristics (which will likely result in adverse toxicity profiles) can be eliminated using the DOLCE assay. Binding of hit compounds to targets can be further interrogated using tandem mass spectrometry techniques (MS/MS) and ion mobility spectrometry[90,91] (IMS) to gain additional insight into the location of ligand binding, as well as the affinity, specificity and stoichiometry of binding. Compounds with favourable solubility and binding profiles can be advanced to 'lead' status and placed in initial biological assays. DOLCE, detection of oligonucleotide–ligand complexes by electrospray ionization; IRMPD-MS, infrared multiple photon dissociation mass spectrometry.

Table 1 | **Mass spectrometry techniques used in the drug discovery process**

| Technique | Methodology | Application | Advantages | Disadvantages | References |
|---|---|---|---|---|---|
| Multitarget affinity/ specificity screening (MASS) | Direct mass spectrometric detection of noncovalent drug–target complexes | High-throughput primary drug discovery screen | Can identify low-affinity compounds missed by traditional screens | HTS/target size is limited by mass range of mass spectrometer | 19,74,75 |
| Structure–activity relationship by mass spectrometry (SAR by MS) | Determination of competitive, cooperative and concurrent ligand binders to a target | Discovery of building blocks that can be linked together to form better binding ligands | Rapid way to optimize ligands using SAR | Provides less information about ligand-binding sites than SAR by NMR | 78,79 |
| Detection of oligonucleotide–ligand complexes by electrospray ionization mass spectrometry (DOLCE-MS) | Direct mass spectrometric detection of non-covalent complexes between ligand and single-stranded and double-stranded oligonucleotides | Differentiating between true hits and false-positives in traditional biological assays | A fast, robust and sensitive technique | Compound solubility can limit applicability | 70 |
| Nanospray mass spectrometry | Use nanospray ionization in place of standard electrospray ionization | Same general applications as conventional electrospray ionization | No sample-to-sample carryover, small sample volumes (1–3 μl) | Cost per sample can be prohibitive for HTS | 83,85-88 |
| Dissociation constant ($K_d$) determination | Use mass spectrometry to determine relative amounts of free and bound targets for calculation of $K_d$ values | Determine absolute dissociation constants for rank ordering of ligands | High-throughput $K_d$ determination possible | Limited use as best results obtained for ligands that interact electrostatically with the targets | 17,20,25,48,51 |
| Collisionally activated dissociation mass spectrometry (CAD-MS) | CAD of complexes | Provides structural information about complexes as well as structure and identity of ligands | Useful for differentiation of ligands with similar mass and binding modes, and the determination of binding sites | Only charged dissociated ligands detected | 30,37 |
| Infrared multiple photon dissociation mass spectrometry (IRMPD-MS) | IRMPD of complexes | Provides structural information about complexes as well as ligands | Useful for differentiation of ligands with similar mass and binding modes, and the determination of binding sites | Only charged dissociated ligands detected | 46,47 |
| Ion mobility mass spectrometry (IMS) | Use ion drift times to determine cross section of complexes | Information about target conformation and ligand binding site(s) | Provides structural information about complexes | Requires fairly sophisticated theoretical modelling | 90,91 |
| Stored waveform inverse Fourier transform (SWIFT) | Complex excitation of ion trajectories in trapped ion cell | Selection of precursor for MS/MS techniques | Accurate selection technique for isolation of precursor ions | Requires FTICR-MS, which is expensive | 45 |

HTS, high-throughput screening; MS/MS, tandem mass spectrometry.

reduced in the ESI-MS approach, because compounds derived from reaction intermediates, degradation products and other impurities are differentiated by molecular mass (the exception being stereoisomers). Such mixtures can be screened in such a way that interactions between the target and any of the components of the mixture are accurately captured. Importantly, binding affinities and specificities are assigned on a component-by-component basis, not on the basis of the collective behaviour of the mixture. Here, we highlight several bodies of work in which the presence (or absence) of noncovalent complexes as detected by ESI-MS provide key information that contributes to, or drives, primary compound screening activities and structure–activity relationship (SAR) optimization.

**Interrogation of protein targets**

ESI-MS has been used to study the binding of vancomycin group antibiotics to bacterial cell wall peptide analogues[24–30]. These antibiotics cause cell death by binding to peptidoglycan precursors ending in the sequence Lys-D-Ala-D-Ala and inhibiting bacterial cell wall growth[31]. Heck and colleagues expanded the work of Henion and co-workers by showing that the dissociation constants of several different ligands could be measured simultaneously, which facilitates the direct screening of combinatorial libraries[29]. Importantly, dissociation constants derived in that work were in agreement with solution-phase values that were determined individually for each ligand. It should be noted that the vancomycin group antibiotics bind the peptide ligands
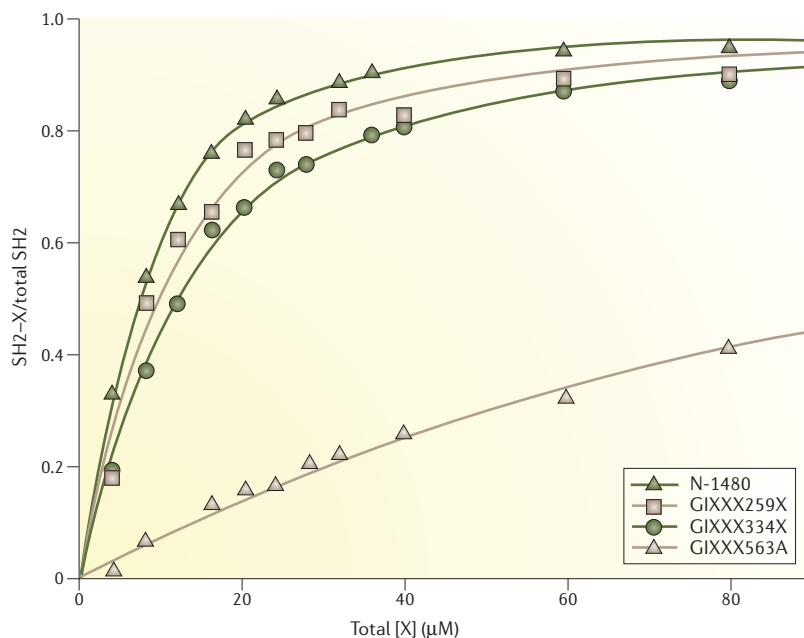
Figure 2 | **Application of ESI-FTICR mass spectrometry in ligand screening. a** | Spectrum acquired from a solution containing 7 μM carbonic anhydrase (CAH) holoprotein (that is, CAH with noncovalently bound Zn(II)) and an assortment of benzenesulphonamide inhibitors with different amino acid constituents. **b** | Following isolation and dissociation, the liberated ligands are directly measured by ESI-FTICR, allowing both identification and quantitation of the inhibitors. **c** | Subsequent isolation and fragmentation of the liberated ligands enabled structural interrogation and discrimination of isobaric ligands. Reproduced, with permission, from REF. 37 © (1995) American Chemical Society. ESI-FTICR, electrospray ionization Fourier transform-ion cyclotron resonance.

cyclotron resonance)[37] (FIG. 2). Libraries containing compounds known to bind carbonic anhydrase with high affinity were mixed in solution with carbonic anhydrase under non-denaturing solution conditions. The intact noncovalent complexes were detected using FTICR-MS. Collisionally activated dissociation (CAD) was subsequently used to dissociate the noncovalent complexes in the gas phase. As shown in FIG. 2b, ligands liberated from both the dissociated noncovalent complexes and the free protein are readily detected in the spectrum, enabling accurate mass measurements and relative abundance measurements of the previously bound ligands. Next, the liberated (charged) ligands can be further analysed by high-performance FTICR measurements[37] (FIG. 2c). The FTICR platform is uniquely suited for this application as it accommodates tandem mass spectrometry (broadband MS/MS of the noncovalent complexes in this instance), highly accurate high-resolution mass measurements of the ensemble of liberated ligands (which allows many compounds to be assayed simultaneously), and additional interrogation of the liberated ligands via mass spectrometry[3]. In their initial work, Smith and colleagues observed a strong correlation between the measured solution-phase $K_d$ of the complex and the gas-phase abundance of the ligands (dissociated from the protein–ligand complexes). Additionally, it was shown that the dissociated ligands could be further interrogated by tandem mass spectrometry, thereby providing structural information about the affinity-selected ligands and offering a method with which to discriminate between members of a combinatorial library that could comprise the same building blocks. Later work demonstrated that the scheme was applicable to more complex libraries and that relative binding affinities derived from these measurements correlated with those derived from conventional methods for determining $K_d$ values[38]. The rank order derived from the relative binding affinities was consistent with that derived from solution-phase measurements[39]. This work demonstrated for the first time that the affinity of multiple potential ligands for a single macromolecular target could be evaluated simultaneously using mass spectrometry, which laid the foundation for subsequent high-throughput screening approaches.

Similarly, Marshall and co-workers used a combination of ESI, gas-phase isolation, gas-phase dissociation and high-performance FTICR measurements to screen a 324-member peptide library for Hck Src homology 2 (SH2) domain ligands[40]. Src SH2 domain protein (~12 kDa) binds phosphorylated tyrosine-containing peptides and is important in the signal transduction pathways of growth factor, cytokine and antigen receptors[41–44]. In addition, poor function of the Src SH2 domain has been associated with cell transformation and cancer[40]. As such, SH2 domains are potential drug targets for a wide variety of biological processes. In the approach of Marshall *et al.*[40], a solution containing the Hck SH2 domain and a 324-member combinatorial peptide library (Ac-GpYEXX-Eda) was ionized by ESI and the resulting noncovalent complexes 'pre-surveyed' by FTICR-MS. The large number of complexes in solution with nearly identical

**Collisionally activated dissociation**
(CAD). Dissociation is caused by collisions between ions and gaseous molecules that result in the conversion of translational energy into internal vibrational energy of the ion.

**SH2 domain**
(Src homology 2 domain). A protein motif that recognizes and binds tyrosine-phosphorylated sequences, and therefore has a key role in relaying cascades of signal transduction.

through several hydrogen bonds and that this type of intermolecular interaction is well preserved in the gas phase[32–36]. However, differences in the binding affinity of a molecule in the gas phase and solution phase have been reported[12,32], which highlights the importance of hydrophobic interactions.

*Single protein targets.* Smith and co-workers demonstrated that noncovalent complexes of various modified benzensulphonamide inhibitors and carbonic anhydrase could be characterized by ESI-FTICR (Fourier transform-ion

Figure 3 | **Application of ESI-MS in determining binding modes.** Plot shows ESI-MS titration graphs for binding of an inhibitor to Src SH2 protein. Increasing concentrations of inhibitor (X) were added to Src SH2 protein (8 μM in 200 mM NH$_4$OAc, pH 5.5) and the mixtures analysed by ESI-MS. The relative amount of complex (SH2–X/total SH2) formed is plotted against concentration. Mass spectrometry showed that one ligand–one protein complex is formed and so a one ligand–one binding site model was used to calculate $K_d$ values. The calculated $K_d$ values for N-1480, GIXXX259X, GIXXX334X and GIXXX563A are 3.2, 5.3, 7.9 and 101 μM, respectively. Reproduced, with permission, from REF. 51 © (2003) Wiley InterScience. ESI-MS, electrospray ionization mass spectrometry.

mass led to a very complex spectrum in which the isotopic envelopes from individual noncovalent complexes overlapped. It was therefore impossible to directly determine which ligands were responsible for the broad hump of noncovalent complexes apparent in the spectrum. To determine which ligands were bound to the target and to establish the relative binding affinities of the ligands, a single charge state of the noncovalent complexes was isolated in the gas phase using the stored waveform inverse Fourier transform (SWIFT) technique, as described in REF. 45. This ensemble, which comprised a single charge state of the noncovalent complexes, was subsequently dissociated by the infrared multiple photon dissociation (IRMPD) technique. Here, an infrared laser is used to generate molecular dissociation[46,47], which facilitates the direct characterization of the ligands that were originally components of the gas-phase noncovalent complexes. In both approaches the relative affinities of the ligands are inferred from their abundance in the gas phase post-dissociation, on the assumption that all the complexes are completely and uniformly dissociated.

In situations in which a relatively large library containing many high-affinity ligands is to be screened, this approach has potential advantages over those in which intact noncovalent complexes are directly measured, because it is difficult, if not impossible, to 'unwind' such complex spectra and determine the relative contribution of each ligand–target pair to the observed

signal. Mixtures of large noncovalent complexes that differ by only a few Daltons will produce spectra in which the isotopic envelopes of the noncovalent complexes have significant overlap that can confound spectral interpretation. Alternatively, because the dissociated ligands are typically tens of thousands of Daltons lighter than the target from which they were liberated, the isotopic complexity of the ligands is significantly less than that of the intact complex, and the contributions from individual ligands which differ in mass by less than a Dalton can be unambiguously discerned.

Several other groups have also studied the noncovalent complexes of Src SH2 domain protein[40,48–51]. Loo and co-workers examined the binding of several phosphopeptides to the Src SH2 domain protein[48]. The dissociation constants of a series of phosphopeptides that are sequence stereoisomers were determined by ESI-MS and were in good agreement (within a factor of 2 or less) with values derived from solution-phase experiments. It was also shown that the peptide with the highest relative affinity (based on solution-phase assays) could be identified from an equimolar mixture of six peptides using ESI-MS. Robinson and co-workers have shown that ESI-MS can be used to examine the role of water in specific interactions between phosphopeptides with Src SH2 and Fyn SH2 domain proteins[49,50]. Lowe and co-workers examined the binding of inhibitors to the Src SH2 domain protein[51] with small-molecule inhibitors that mimic phosphotyrosine. Dissociation constants were determined for each inhibitor by a titration method and by a competitive binding method. Excellent agreement was observed between the two ESI-MS methods, and the ESI-MS-derived values were within a factor of 2 or less of the values derived from the solution-phase experiments. FIGURE 3 shows the ESI-MS titration graphs for four different inhibitors (N-1480, GIXXX259X, GIXXX334X and GIXXX563A) of Src SH2 domain protein. Using mass spectrometry, the relative amount of protein–ligand complex is measured as increasing concentrations of the ligand are added to the solution. For these four compounds, only a 1:1 protein–ligand complex was formed, based on mass spectrometric data, and therefore a one ligand–one binding site model was used to determine the $K_d$ values. A fifth ligand unexpectedly formed a 1:2 (protein–ligand) complex in addition to a 1:1 complex (data not shown). Because the binding stoichiometry for this ligand was evident from the mass spectrum, the data were analysed by a one ligand–two binding site model.

ESI-MS can be used to measure the gas-phase stabilities of protein–ligand complexes, which can then be compared with solution-phase stabilities. Podjarny and co-workers examined the gas-phase stabilities of inhibitors of aldose reductase[34], an NADP(H)-dependent enzyme believed to cause degenerative complications associated with diabetes mellitus[52]. Relative gas-phase stabilities of noncovalent enzyme–coenzyme–inhibitor complexes were determined with CAD-MS. Dissociation of the complex is caused by collisions with the gaseous molecules in the interface region where the pressure is relatively high (1–4 mBar) and the degree of dissociation is controlled by the accelerating cone voltage. The accelerating cone voltage at which 50%

of the noncovalent enzyme–coenzyme–inhibitor complex dissociates ($Vc_{50}$) was used as a measure of the gas-phase stability of the complex. The gas-phase stabilities ($Vc_{50}$) and the solution-phase binding energies ($IC_{50}$) were determined for four noncovalent enzyme–coenzyme–inhibitor complexes. The electrostatic and H-bond energies ($E_{el-H}$) were calculated from crystallographic data for the same four noncovalent enzyme–coenzyme–inhibitor complexes. Although there was a good correlation between $Vc_{50}$ and $E_{el-H}$, no correlation between $Vc_{50}$ and $IC_{50}$ values was identified. This indicates that the gas-phase stabilities measured by $Vc_{50}$ reflect the electrostatic and H-bond interactions of the inhibitor with the enzyme and not the hydrophobic interactions of the inhibitor. It is common to modify the electrostatic and H-bond contacts of inhibitors during the drug development phase, and so the ability to determine the strength of these interactions through gas-phase stability measurements is useful[34]. Other studies using different protein–ligand systems have produced similar results — that is, in the gas phase, electrostatic and H-bond interactions are favoured over hydrophobic interactions[33,35,53].

*Multimeric protein targets.* The studies described above focused on ligand binding to a single protein target. It has also been shown that mass spectrometry can be used to study ligand binding to a multi-protein target[54–56]. Using ESI-MS, Robinson and co-workers examined 18 ligands that bound to the hormone-binding protein transthyretin in its homotetramer state[54] (transthyretin is interesting because it can be deposited as amyloid fibrils that are associated with Alzheimer's disease and spongiform encephalopathies)[57,58]. The authors were able to rank order the binding of the 18 ligands and identified *N*-phenyl phenoxazine derivatives as the strongest binders, which correlates with measurements from turbidity assays[59]. However, there were differences in the rank order of the derivates between the two methods, which were partly attributed to the different concentrations of ligand used in the two methods and to differences in the hydrophobicity of the compounds. It was also shown that the inhibitors increase the stability of the transthyretin tetrameric conformation and do not prevent the binding of retinal-binding protein, which is necessary for transthyretin to transport vitamin A in humans. The ability to evaluate ligand interactions with multi-protein systems broadens the applicability of mass spectrometry to studying even more complex systems that have biological relevance.

*Off-target proteins.* In addition to using mass spectrometry to identify potential drugs that noncovalently bind proteins that cause disease, mass spectrometry can be used to determine whether and where potential drugs noncovalently bind to off-target proteins, such as human serum albumin (HSA)[60]. Drug binding to HSA lowers the active concentration of the drug, as well as affecting the pharmacokinetic, pharmacodynamic and toxicological properties of the drug. Using separate competition experiments with warfarin (site I binder), iopanoic acid (site II binder) and digitoxin (site III binder), it was determined that two compounds specifically bind

to the warfarin binding site of HSA[60]. This is just one example of the valuable information that this technique can provide early in the drug discovery process.

## Analysis of DNA–ligand complexes

Although proteins are the central focus of the vast majority of drug discovery strategies, alternative targets based on DNA and RNA are gaining prominence as they often offer access to otherwise 'undruggable' targets. Compounds that interact directly with DNA can prevent tumour or microorganism growth, or inhibit gene expression by preventing binding of necessary cofactors. Such drugs can bind to single-stranded DNA, duplexed DNA and higher-order structures of DNA. Such DNA binders are grouped into two classes: minor-groove binders and intercalators. Minor-groove binders typically interact with DNA through extensive H-bonding, are often positively charged and generally have a preference for AT-rich sequences, whereas intercalators insert between the stacked base pairs of the DNA and interact with DNA through hydrophobic effects and van der Waals forces. Therefore, rapid and robust screening methods that can determine the binding stoichiometry, specificity and affinity of lead compounds to DNA could have an important role in the drug discovery process.

Several groups have investigated the binding of known minor-groove binders and intercalators to duplex DNA[61–69]. De Pauw and co-workers studied the binding of five minor-groove binders to five different duplexed DNAs that contained 12 base pairs with varying AT compositions[62]. Four out of the five minor-groove binders (Hoechst 33258, Hoechst 33342, DAPI and berenil) formed 1:2 DNA–drug complexes in addition to 1:1 complexes. Under the same conditions, Netropsin was observed to form exclusively 1:1 complexes with DNA, indicating that the 1:2 complexes observed with the other drugs are specific and not due to nonspecific aggregation. These investigations also showed that mass spectrometry can be used to assess the preference of drugs for binding to sequences with subtle difference, such as ATAT compared with AATT sequences.

*DOLCE-MS.* As illustrated above, ESI-MS can be used to identify ligands that bind to specific targets and to study aspects of key interactions such as affinity, specificity and stoichiometry. Interestingly, there are instances in which it is advantageous to rule out 'off-target' binding events that have the potential for downstream toxicity or carcinogenicity. Greig and Robinson developed the high-throughput screen DOLCE-MS (detection of oligonucleotide–ligand complexes by electrospray ionization mass spectrometry)[70]. In this approach multiple ligands can be screened simultaneously against single-stranded and duplexed oligonucleotides at a rate of 5 min per sample, allowing ~200 samples to be screened in a single day. This approach has several potential advantages over other techniques such as NMR, circular dichroism, gel-shift assays and surface plasmon resonance. Less sample material is required, analysis times are shorter, and data interpretation is more straightforward compared with the other 'conventional' techniques. This approach was
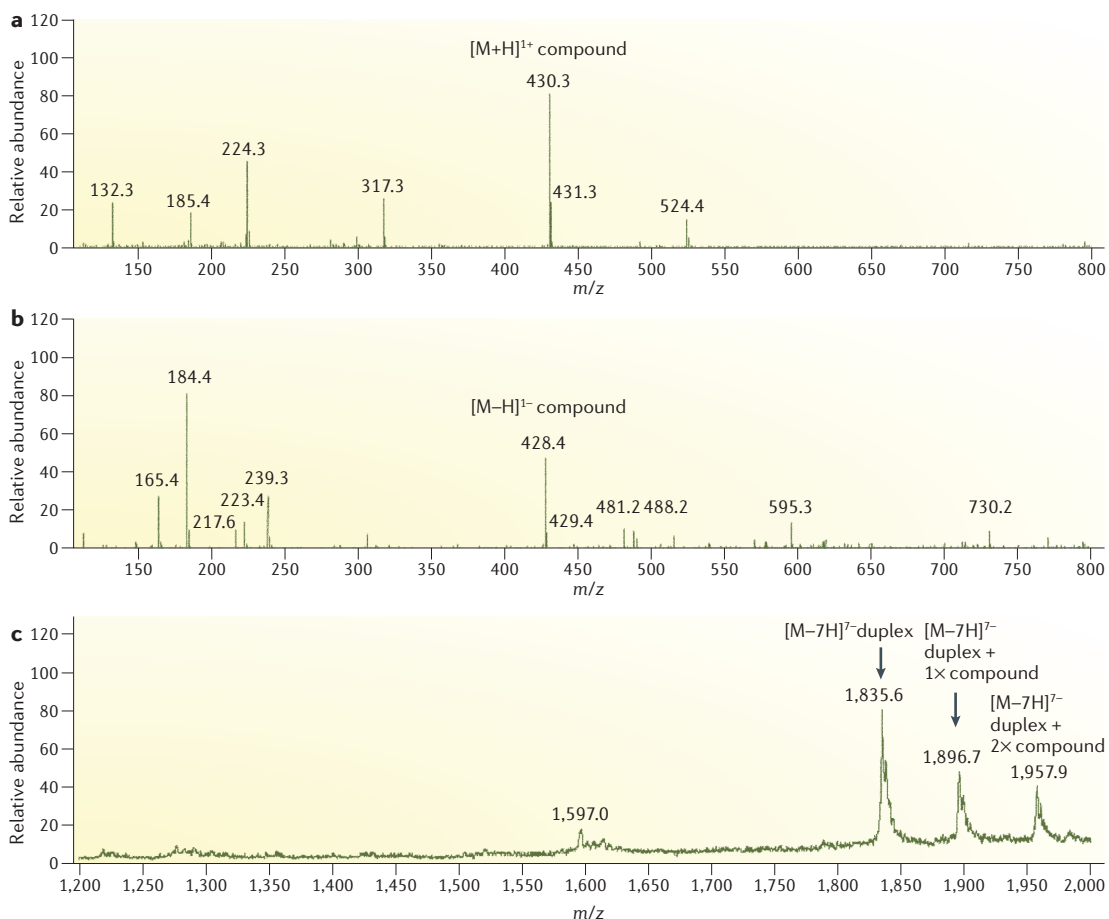
Figure 4 | **Use of DOLCE-MS to determine binding characteristics of a compound.** Spectra shown are from DOLCE-MS analysis of a compound that binds to duplex DNA. ESI mass spectra of the drug candidate (molecular mass of 429.3 Da) were first recorded in positive ionization mode (**a**) and negative ionization mode (**b**): the signal was averaged for 30 seconds. Both **a** and **b** show that the compound was the major product synthesized. The high-molecular-mass region of the negative ionization mode mass spectrum depicted in **c** (multiply charged state) was acquired in the presence of a DNA substrate and clearly shows that the compound binds the duplex DNA target at two or more sites. Reproduced, with permission, from REF. 70 © (2000) SAGE. DOLCE-MS, detection of oligonucleotide–ligand complexes by electrospray ionization mass spectrometry; ESI, electrospray ionization.

recently applied to support a drug discovery effort in which the intended protein drug target was known to interact directly with duplex DNA[70]. DOLCE-MS was used to identify compounds with high affinity for DNA, and a concomitantly high toxicity potential, so that they could be eliminated from consideration early on in the drug discovery process (FIG. 4).

*RNA–ligands.* Because RNAs perform various crucial functions in the cell, RNA is an attractive target for therapeutic intervention in various diseases, including bacterial and viral infections, metabolic disorders and cancer. RNA serves as the intermediary transcript between genomic information encoded in DNA and gene products (proteins). RNA-based structural elements are central to cellular and viral life, including alternative splicing, gene regulation, recognition and protein translation in the ribosome[71]. For example, little or no change can be tolerated at active sites in ribosomal RNA (rRNA) and at protein-binding regions of mRNA.

Recent advances in the determination of RNA structure and function make targeting unique RNA motifs with small molecules a tractable problem[72,73].

*MASS.* We have developed a mass spectrometry-based approach for the discovery of small-molecule ligands that bind to structured regions of RNA. This approach is called multi-target affinity/specificity screening (MASS) and uses ESI-FTICR-MS[19,74,75]. In a single assay, MASS can determine the chemical composition of ligands that bind to an RNA target, the relative/absolute dissociation constants, and the specificity of binding to one RNA target relative to other RNA targets. Ligand–RNA complexes with affinity ranging from 10 nM to greater than 1 mM can be observed in the MASS assay[5,19,74,75]. This technology has been used to evaluate the binding of ligands to several RNA targets, including the 16S ribosomal RNA A-site, internal ribosome entry site IIA subdomain of the hepatitis C virus, and 1061 region of the bacterial 23S rRNA[76–79].
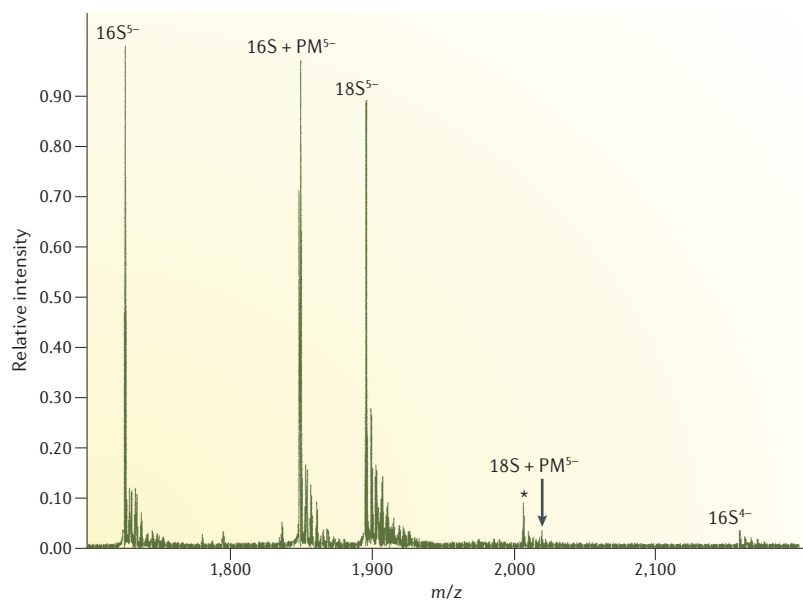
Figure 5 | **Application of MASS in the identification of small-molecule ligands that bind to structured regions of RNA.** The mass spectrum shown is of one titration point in the simultaneous determination of the dissociation constant ($K_d$) values. $K_d$ values were determined for paromomycin (PM) with 16S and 18S. 16S and 18S are both at 100 nM concentration and the paromomycin concentration is 250 nM. The 5– charge state (that is, $[M-5H^+]^{5-}$) of the free RNAs and the complexes are shown. The abundance of the 16S + PM complex relative to the free 16S compared with the abundance of the 18S + PM complex relative to the free 18S indicates that PM has a higher affinity for 16S than for 18S. The asterisk marks an impurity in the spectrum. MASS, multi-target affinity/specificity screening.

The most common noncovalent interactions in biology are low affinity and are difficult to detect directly by traditional analytical methods. Identification of low-affinity ligands ($K_d$ values of >100 μM) and information about their binding sites can provide information for the design of higher-affinity ligands. Low-affinity ligands are identified with the MASS assay in a robust and rapid manner. Griffey et al.[18] demonstrated that 2-deoxystreptamine (2-DOS) binds to the 27-mer prokaryotic 16S rRNA A-site construct (16S) at multiple locations with $K_d$ values ranging from 600 μM to 15 mM. Molecular modelling showed that there were two high-probability binding sites for 2-DOS on the 16S subunit and that there were additional higher-energy binding sites. This indicates that low-affinity ligands can bind specifically to a target[18]. In addition, it was demonstrated that the MASS assay could be used to examine concurrent and competitive binding of low-affinity compounds[18]. For example, 2-DOS and 3,5-diamino-triazole (3,5-DT) were shown to bind concurrently to 16S, suggesting that 2-DOS and 3,5-DT bind the 16S target in different locations. Alternatively, ESI-MS data suggest that 2,4-diaminopyrimidine (DAP) and 2-DOS bind 16S competitively, because DAP was not observed to bind the 16S construct in the presence of 2-DOS. It was therefore argued that 2-DOS and DAP bind to 16S in the same or overlapping locations. This information can be used to build ligands from small motifs and thereby increase ligand affinity and specificity for a drug target.

For the MASS approach to be a high-throughput assay, it must be possible to screen multiple targets against multiple ligands in a single well. This capacity requires that the molecular interaction between any given target–ligand pair is independent of the presence (or absence) of other ligands and targets in solution. In previous work[75], we demonstrated that lividomycin specifically binds to the 27-mer 16S A-site construct in the presence of two other RNA targets and 25 non-binding compounds that are present at a 400× molar excess relative to the amount of lividomycin. As is common practice in our laboratory, three targets at 2.5 μM (each) were screened against 11 ligands, each of which has a concentration of 25 μM. The RNA concentration ensures that there is enough RNA available for all potential binders to interact with the RNA. The relatively high ligand concentration ensures that even ligands with $K_d$ values of the order of 1 mM will be detected. Typically, one well is screened every 39 seconds (~1 plate per hour): 33 seconds of data acquisition (20 co-added scans) and 6 seconds of overhead associated with the autosampler. This means that in a 24-hour period, 22,500 compounds are screened against 3 targets, resulting in the interrogation of ~67,000 potential ligand–target interactions (for a detailed description, see REF. 75).

MASS is not only useful for finding new compounds that bind the target with high affinity, but also for thoroughly characterizing the binding properties of ligand–target pairs to better understand the specificity of a given interaction. For example, using the MASS assay, the $K_d$ values for the aminoglycoside antibiotics paromomycin and tobramycin were determined against 16S and a 27-mer construct of the eukaryotic 18S rRNA A-site (18S). Both 16S and 18S were held at 100 nM while the aminoglycoside antibiotics were screened at concentrations of 250 nM, 750 nM, 2.5 μM and 7.5 μM. FIGURE 5 shows an example titration point for the $K_d$ determination of paromomycin against 16S and 18S. The $K_d$ values for 16S determined for paromomycin (132 nM) and tobramycin (352 nM) are consistent with solution-phase determinations[8,80] and our previous ESI-MS measurements[20]. The $K_d$ values for 18S determined for paromomycin (1.55 μM) and tobramycin (266 nM) show that paromomycin binds preferentially to 16S, whereas tobramycin has equal preference for binding to 16S and 18S. A total of 380 seconds of data acquisition is needed to collect the four concentration points for each $K_d$ determination, and so the MASS assay provides a robust way to determine $K_d$ values for >100 compounds a day.

We recently extended the MASS assay to the screening of natural product broths[81]. In a proof of principle study, fractionated broths from *Streptomyces rimus paromomycinus*, which is known to produce the aminoglycoside paromomycin, were screened against a 27-mer 16S A-site RNA construct (16S) with a three-nucleotide internal bulge, and a 28-mer control RNA construct (16Sc), in which the 16S construct was modified by replacing the internal bulge with Watson–Crick base pairs. By screening against these two targets simultaneously, ligands that bound specifically to the internal bulge of 16S were identified, as were non-specific ligands that bound equally well to both constructs (based on the ratio of the 16Sc and 16S complexes).
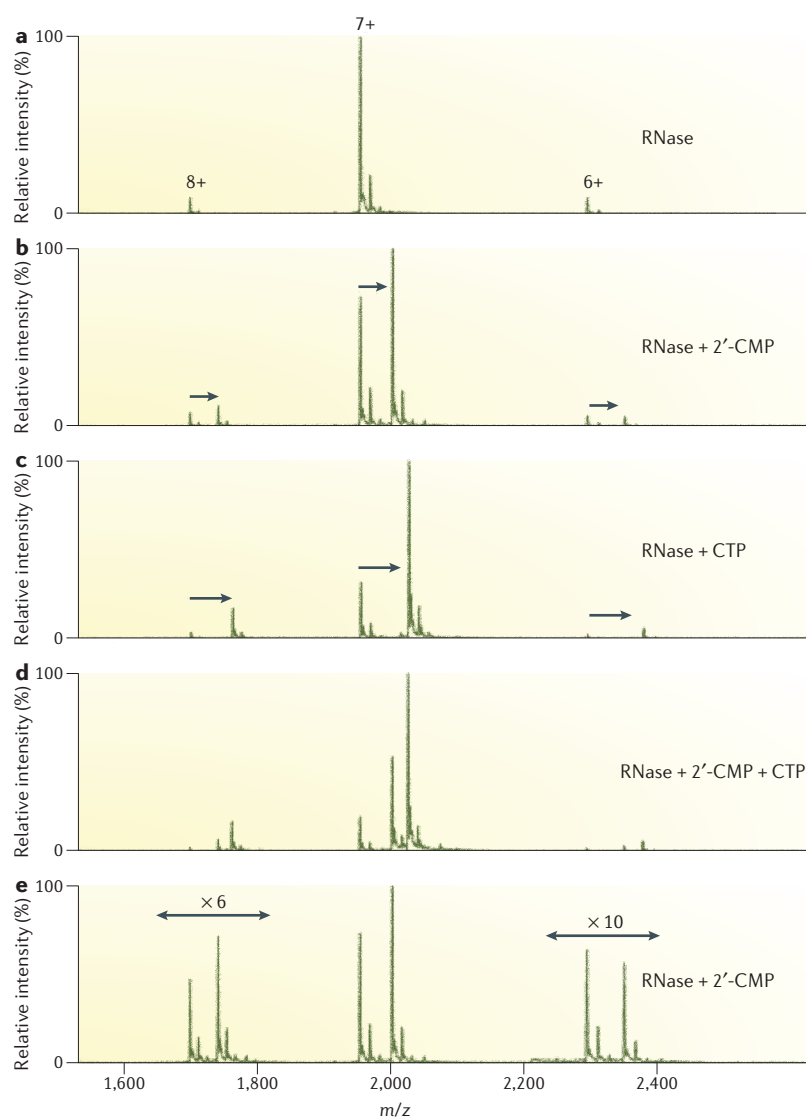
Figure 6 | **Mass spectra of RNase A complexed with 2′-CMP and CTP.** Spectra were obtained in ammonium acetate (10 μM, pH 6.8) and generated using nanospray in a fully automated modality. The use of the nanospray platform eliminates sample-to-sample carryover, which can confound the sequential analysis of closely related compounds. **a** | Mass spectrum of RNase A (10 μM). **b** | RNase A (10 μM) plus 2′-CMP (10 μM). **c** | RNase A (10 μM) plus CTP (10 μM). **d** | RNase A (10 μM) plus 2′-CMP (10 μM) and CTP (10 μM). **e** | Same data from panel **b** but scaled to show that the ratio of free RNase A ion intensity to RNase A-2′-CMP complex ion intensity varies with charge state. Reproduced, with permission, from REF. 87 © (2003) American Chemical Society.

single structure with higher affinity for the target. Griffey and co-workers used this technique to identify motifs for the internal ribosome entry site IIA subdomain of hepatitis C virus[78] and the 1061 region of the bacterial 23S ribosomal RNA[79]. In the latter example, two different classes of motifs were identified that generated profiles of concurrent, competitive and cooperative binding for the specific region of the bacterial RNA. By linking the motifs together, a rigid biaryl linked compound was constructed that displayed a 20-fold enhancement in affinity for the RNA target relative to the individual motifs. A variation on the SAR-by-MS approach has also been applied to the protein target stromelysin, which identified novel inhibitors of this protein[82].

**Variation on a theme: nanospray**
The studies described above used 'conventional' ESI in which samples are infused at a rate of approximately 1–25 μl per minute through a reusable electrospray needle with an internal diameter of typically 50–150 μm. A variation on this format, termed nanoelectrospray or nanospray, uses a smaller-diameter ESI emitter (typically 2–20 μm) and offers several attributes that could be advantageous to certain facets of ESI-MS-based drug discovery[83]. First and foremost, significantly less sample is consumed in an analysis, with meaningful measurements being made from total sample volumes in the 1–3 μl range. Moreover, it has been argued that nanospray is gentler than ESI in a conventional format[50,84]. Nanospray has been shown in some instances to provide greater sensitivity than conventional ESI (based on both less sample consumption and more efficient ion desolvation) and to be more tolerant of nonvolatile cations in solution[83,85–87].

Recently, a microfabricated chip-based nanospray platform has been introduced that overcomes some of the difficulties associated with using nanospray in a routine and robust manner. The automated chip-based nanospray system has been used to determine dissociation constants for two ligand–protein systems, to screen multiple ligands against a protein and to examine ligand binding to a multimeric protein complex[86–88]. Titration and competition experiments using the nanospray chip gave dissociation constants for 2′-CMP and CTP to ribonuclease A that are consistent with literature values derived from solution-phase methods[87]. It was noted that the ratio of ligand bound to ribonuclease A was dependent on the charge state of the complex (FIG. 6). This observation was rationalized by the hypothesis that ligand binding induces a conformational change in the protein, which in turn affects the range of preferred charge states of the complex. This difference was accounted for by integrating all charge states in the dissociation constant calculations. In other work the ESI chip was used to determine dissociation constants for four oligosaccharide ligands to an inactive endocellulase mutant (endocellulase hydrolyses cellulose to cellobiose and glucose, and has potential commercial value for producing ethanol)[89]. Accurate determination of dissociation constants among closely related compounds and targets can provide important information pertaining to specificity of binding and can provide insights into further optimization of lead compounds. In this instance,

*SAR by MS.* Building on MASS, we developed SAR by mass spectrometry (SAR by MS) as a ligand-based lead discovery strategy[78,79]. An initial panel of small molecules consisting of various chemical motifs is screened against a target using MASS. Motifs that bind the target at different locations are evident as ternary complexes in the mass spectrum. Simple derivatives of the most interesting motifs are made that will help to identify potential linkage sites between motifs and then rescreened using MASS. These data afford new insights into motif-binding sites and orientation on the target. Using this information, several motifs can then be linked together into a

the mass spectrometry-based method was able to determine values for cellotriose, cellotetraose and cellopentaose, as well as estimate the value for cellohexaose[87]. Finally, the nanospray chip was used to investigate ligand binding to the tetrameric plasma protein transthyretin[86]. The binding of two thyroxines to transthyretin was detected. It was clearly demonstrated that the nanospray chip gave reproducible spectra, which enabled comparison between multiple spectra and determination of relative binding affinities across multiple spectra. Although a number of compelling results have been obtained on this integrated microfabricated chip-based platform, it remains to be seen how practical this platform will be in a true 24/7 high-throughput screening modality when consumables cost per sample and analysis time per sample are often key value drivers.

## Past experience and future directions

It is clear from the examples highlighted above, and from many more referenced above, that the utilization of mass spectrometry in the drug development process is changing and is increasingly expanding into earlier stages of lead discovery and compound optimization. The commercial availability of increasingly sophisticated high-performance mass spectrometers (such as the quadrupole-FTICR, and the linear ion trap-FTICR), and the falling costs and improved performance of benchtop systems (for

example, ESI-time of flight, linear ion trap, high-capacity quadrupole ion trap), have put the tools necessary for ESI-MS analysis of noncovalent complexes in the hands of many drug discovery researchers. It is likely that the broad availability of these capabilities will create another 'wave' of ESI-MS applications that interrogate noncovalent complexes, which will support existing strategies in drug discovery, and enable the development of new ones.

In addition to the mass spectrometric techniques described, other gas-phase methods for probing the structure of biomacromolecules and noncovalent complexes could have key roles in the drug discovery field. For example, ion mobility spectrometry is re-emerging as a method to rapidly interrogate higher-order structure of macromolecule and noncovalent complexes in the gas phase[90,91]. Such approaches might provide drug discovery researchers with new insights into ligand–target interactions at the molecular level at earlier stages in the drug discovery process, allowing the development of compounds with undesirable binding characteristics to be terminated earlier and at less overall expense. The very small quantities of targets and ligands required, the ease with which these assays can be automated, and the broad applicability to diverse target classes bode well for continued (and expanded) use of ESI-MS to characterize noncovalent complexes as a flexible platform to support the ever-changing landscape of modern drug discovery.

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**Competing interests statement**
The authors declare no competing financial interests.