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SPECIAL FEATURE: TUTORIAL

Electrospray Ionization Mass Spectrometry for the Study of Non-covalent Complexes: an Emerging Technology

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The detection of non-covalent complexes in the mass range 19 000–34 000 Da, using electrospray ionization mass spectrometry (ESI-MS), is reviewed. The examples discussed include (1) a protein–ligand interaction (*ras*–GDP), (2) an inhibitor–protein–ligand interaction (SCH 54292/SCH 54341-*ras*–GDP), (3) a protein–protein interaction (γ-IFN homodimer) and (4) a protein–metal complex [HCV (1–181)–Zn]. In each case, the ESI-MS method is capable of releasing the intact non-covalent complex from its native solution state into the gas phase in the form of multiply-charge ions. The molecular masses of these complexes were determined with a mass accuracy of better than 0.01%, which is far superior to the traditional methods of sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel permeation chromatography. The method provides the researcher with a quick, reliable and reproducible method for probing difficult biological problems. The key to success in the study of non-covalent complexes depends on careful understanding and manipulation of ESI source parameters and sample solution conditions; special care must be taken with the source orifice potential and the solution pH and organic co-solvents must be avoided. This paper also illustrates the usefulness of ESI-MS for addressing biological problems leading to the discovery of new therapeutics; the approach involves the rapid screening of potential drug candidates, such as weakly bound inhibitors. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: non-covalent complexes; electrospray ionization mass spectrometry; drug discovery

INTRODUCTION

Cellular function is often triggered by weak non-covalent interactions between enzyme and substrate, protein and ligand, protein and protein and antibody and antigen. A few specific examples of important non-covalent complexes are *ras*–GDP (protein–ligand), gamma-interferon (γ-IFN) (protein–protein homodimer), farnesyl–protein transferase (FPT) (heterodimer of two subunits), hepatitis C virus (HCV) (protein–metal–peptide complex), FKBP–rapamycin (protein–inhibitor complex) and HIV-1 protease (homodimer of two subunits). Abnormalities can arise from interruption of these normal interactions and lead to disease.

These systems and many others are under intensive investigation to understand better how the human body functions or, more importantly, malfunctions and how best to prevent the alteration of normal processes by disease. The malfunctioning of the *ras* protein is an example of the disease process. The *ras* protein exists in the body in two interconvertible states: the *ras*–GDP (guanosine diphosphate) complex, which is the inactive state, and the *ras*–GTP (guanosine triphosphate) complex, which is the active state that signals cell growth and differentiation. In oncogenic *ras*, the rate of hydrolysis by the GTPase activating protein that triggers the conversion of the GTP ligand to GDP is dramatically reduced; this 'locks' the *ras* protein in the active state, resulting in unregulated cell growth.

Nearly 20% of all human tumors are linked to malfunctions of this *ras* oncogene (cell growth on–off switch), with the largest distribution found in pancreatic (90%), colon (50%) and lung (40%) cancers.[1] Greater understanding of the structures of these complexes will provide an insight into the mechanism of enzyme interactions and how they control bodily functions. This knowledge may assist the researcher in the discovery of small molecules to act as inhibitors to the disease process, which is an important part of the modern drug discovery effort.

The conventional techniques used for direct detection of non-covalent complexes [e.g. gel permeation chromatography (GPC), centrifugation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)] provide limited or no information about the molecu-

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lar mass and binding stoichiometry of the complex. Advanced spectroscopic techniques such as x-ray and NMR methods have been used to solve the 3-D structures of various proteins, but direct proof about non-covalent complexes based on molecular mass information is lacking. Mass spectrometry (MS), with recent advances in ionization methods, can provide an alternative approach for the study of non-covalent complexes, an approach that directly provides the molecular mass of the complex.

To perform direct MS measurements on pre-formed non-covalent complexes successfully, the MS ionization technique employed must satisfy the following criteria: (i) the ionization method must be capable of generating intact gas-phase protein ions from solutions which contain biological buffers; (ii) the internal energy transferred to the macromolecule during the ionization process must be low enough to prevent dissociation of the complex; and (iii) the MS instrumentation must have a sufficient mass range to observe the ionized complex. In the last 10 years, two ionization techniques have been developed that fit the above criteria, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).

In recent years, the technique of ESI[2–4] has become an important tool in the field of biomedical research involving protein and peptide structural analysis. ESI generates charged droplets at atmospheric pressure by spraying the sample solution under a strong electric field. Ionization occurs by protonation to produce gas-phase macromolcular ions directly from solution. This ionization process is very soft and leaves the ion largely unfragmented; hence complexation involving non-covalent interactions can be studied. The ESI experiment can be carried out in aqueous solution (near physiological conditions) without the presence of an organic cosolvent that tends to denature proteins. Owing to the presence of basic amino acid residues in proteins, multiple proton attachment occurs, resulting in the formation of multiply-charged ions. The net result is that the mass/charge ratio, measured in MS, is greatly reduced, allowing proteins and their complexes as large as 30 kDa or higher to be measured with a modern quadrupole mass spectrometer.

MALDI has also been demonstrated to be an important method for structural characterization of proteins.[5,6] With the use of a time-of-flight analyzer, the MALDI technique is able to detect biomolecules as large as 500 000 Da. The MALDI technique is an extremely sensitive method, requiring low-femtomole amounts of material; the sensitivity achieved with MALDI is more than 10 times that of ESI. Although reports on the use of the MALDI technique for the study of non-covalent complexes are very limited, this methodology appears to be applicable to the detection of non-covalent complexes, at least those of higher binding energies.[7,8] MALDI in combination with chemical cross-linking has been used to assess quantitatively protein quarternary structure to provide the molecular mass of the intact complex and of the individual subunits.[9a] In a recent review, Farmer and Caprioli[9b] provided an overview of various strategies for the determination of protein–protein interactions by MALDI; topics discussed include specific matrix and laser combinations, accumulation of 'first shot' spectra, modification of pH and solvent conditions and use of cross-linking agents.

The first applications of ESI for the detection of non-covalent complexes were reported by Ganem *et al.*[10,11] They studied the binding between FKBP, an immunosuppressive protein, and the immunosuppressive agents FK506 and rapamycin, and, in a second paper, the enzymatic reaction of hen egg white lysozyme with various substrates. This was closely followed by a paper by Katta and Chait[12] studying the heme–globin complex in myoglobin, Baca and Kent's[13] work on the ternary complex of dimeric HIV-1 protease and a substrate base inhibitor and studies of small oligonucleotide duplexes by Smith and co-workers.[14] Subsequently, other reports on the use of ESI to study non-covalent complexes have become common and reviews of this subject have been published recently.[15,16]

In our laboratory, we have been involved in developing MS methodologies for the study of macromolecular non-covalent complexes in order to develop new anti-cancer agents, in addition to supporting other biotechnology programs. Our strategy is to screen potential inhibitors from synthetic or natural sources that bind non-covalently with the disease-causing protein and so interfere with the disease mechanism. Since our initial involvement with this project in 1990, we have developed ESI-based methodologies for the detection of the non-covalent interaction of *ras* protein with its ligands GDP[17] and GTP.[18] We have extended our studies to probe protein–protein interactions with considerable success[19] and in recent work[20,21] have used ESI to detect ternary complexes of *ras*–GDP with the organic inhibitors SCH 54292 and SCH 54341. In the study involving the ternary complex, *ras*–GDP–SCH 54292, we have also identified the drug binding site on the *ras* protein.

In this tutorial, we will provide a detailed description of the ESI-MS methodologies used for the study of non-covalent complexes. Examples of non-covalent complexes cited in this paper are all from work performed in the authors' laboratory and cover a full range of non-covalent interactions, from weak to stronger binding energies. The following examples will be examined: a protein–ligand interaction (*ras*–GDP), a drug–protein–ligand complex [*ras*–GDP–SCH 54292 (also SCH 54341)], a protein–protein interaction (γ-IFN dimer) and a protein–metal complex [HCV (1–181)–Zn]. We also include a brief discussion illustrating the ESI-MS determination of the drug–protein binding site and the use of ESI-MS for screening potential inhibitors for enzymes. The objective of this paper is to provide a simplified description of ESI-MS methodologies that might be useful as a guide for other scientists involved in this type of research.

EXPERIMENTAL

Electrospray mass spectrometry

The experimental results described in this paper to illustrate the use of ESI-MS for the study of non-covalent

complexes were obtained using Micromass Quattro LCZ, Perkin-Elmer (PE) SCIEX API III and PE SCIEX API 365 triple-quadrupole mass spectrometers.

Ras–GDP non-covalent complex

The Micromass Quattro LCZ, equipped with a Micromass Z spray ion source, was used for the *ras*–GDP study. A 20 μM aqueous solution of *ras*–GDP in 2 mM ammonium acetate (pH 5.2) was used. The analyte samples were infused into the mass spectrometer using a Harvard syringe pump (Model 2400-011) at a rate of 2 μl min^{-1} and passed through a 100 μm (i.d.) fused-silica capillary. The mass spectrometer was scanned at a rate of 10 s per scan with the resulting positive ion spectra produced by averaging 2.1 min of accumulative data. The electrospray source conditions used were a sample orifice potential of 70 V, a source temperature of 60 °C and a desolvation temperature of 80 °C. Deconvolution of the *ras* spectral data was performed on a Micromass computer system equipped with their Mass Lynx software using the deconvolution algorithm maximum Ent (entropy) electrospray.

Inhibitor–*ras*–GDP/γ-IFN homodimer

A PE SCIEX API III triple quadrupole mass spectrometer equipped with a standard atmospheric pressure ionization source was used for these experiments. The analyses were carried out by scanning the first or third quadrupole from 300 to 2400 Da per unit charge at a typical scan rate of 2 s per scan. Sample solutions were introduced by either direct infusion of the protein solution or via a Rheodyne external loop injector with a preselected solvent system. In later experiments, an Applied Biosystems Model 140A dual-syringe micro-LC pump was used to deliver constant liquid flow-rates in the range 3–100 μl min^{-1}. The *ras*–GDP and inhibitor were mixed in a molar ratio of 1:10 to prepare the complex solution. For the γ-IFN study, the experiment was performed at 30–35 μM protein concentration.

For experiments (binding site work) involving liquid chromatography/mass spectrometry (LC/MS), a C_{18} microbore LC column using an ABI 140B dual-syringe pump (more recently a Shimadzu pump) was used to introduce samples into the PE SCIEX API III instrument. A Rheodyne biocompatible injector was used for sample introduction on to the microbore column. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) (A) and 0.1% TFA in acetonitrile (B), and a linear gradient from 5 to 75% B in 45 min at a flow-rate of 40 μl min^{-1} was used for separation. The entire eluent was introduced into the mass spectrometer via an articulated ionspray interface. The PE SCIEX instruments were operated at unit mass resolution with a scan rate 3 s per scan over a mass range of 300–3000 Da.

HCV–Zn complex

ESI experiments were performed using a PE SCIEX 365 instrument equipped with a standard atmospheric pressure ionization source. The analyte samples were infused into the mass spectrometer source using a Harvard syringe pump (Model 2400-011) at a rate of 5 μl min^{-1} through a 100 μm i.d. fused-silica capillary.

RESULTS AND DISCUSSION

It is well known that the conformation of a protein in solution is highly dependent on the solvent system used. Proteins generally maintain their native conformation if physiological conditions are approximted. Denaturization of the protein from its native state can be achieved by the addition of acid or an organic solvent or by the application of heat. Over the years, we have found that the detection of non-covalent complexes is highly dependent on the following parameters: solution pH, the presence of organic solvents, the presence of buffer additives, the orifice potential, the source temperature and the sample flow-rate. Therefore, for the successful detection of non-covalent complexes by MS, one must carefully control the solvent system and instrumental conditions.

The ESI mass spectrum of a protein shows a series of protonated multiply charged ions with the charge distribution states reflecting the conformational states[22–24] of the protein; a broad charge distribution shifting the signal to lower m/z values corresponds to the denatured state, while a compact charge distribution at higher m/z values corresponds to the native state of the protein or the complex in solution. The denatured state of a protein exposes more sites for protonation, resulting in the formation of higher charge states.

Protein–ligand interaction

The first application to be discussed, for the use of ESI for the study of non-covalent binding, is a protein–ligand interaction exemplified by the *ras*–GDP system. The decomposition constant, K_D, for the *ras*–GDP system is 20.3 pM (the smaller the value, the stronger is the binding). Figure 1 shows the ESI mass spectrum of a 0.5 μg μl^{-1} *ras*–GDP complex in 2 mM ammonium acetate–0.5% acetic acid (1:1) solution, pH 2.5. Under these conditions, the ESI mass spectrum showed a broad distribution of multiply-charged ions, m/z 2096, 1886, 1715, 1572, 1451, 1348, 1258, 1180 and 1110, with charge states ranging from +9 to +17. The deconvoluted mass spectrum yielded an average molecular mass of 18 854 Da, which is in good agreement with the theoretical molecular mass of 18 853.3 Da for the free apo-*ras* protein. The deconvolution program represents mathematical conversion of a spectrum consisting of multiply-charged ions into a single peak corresponding to the molecular mass of the biomolecule. Therefore, the *ras*–GDP complex was destroyed and only the denatured *ras* protein was detected in this solution. When using a 50:50 acetonitrile–water as the solvent with pH values in the range 2–2.4, the bell-shaped distribution of ions was shifted even further to lower mass

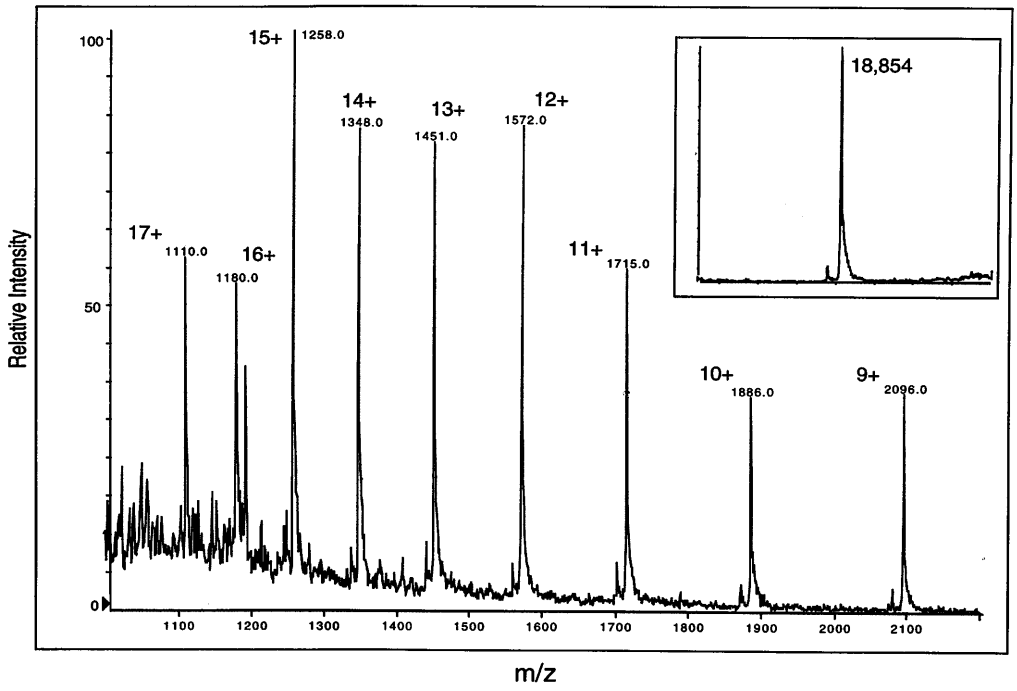

Figure 1. ESI mass spectrum of *ras*–GDP obtained at pH 2.5 in 2 mM ammonium acetate buffer using 20 μM protein solution. Inset shows the deconvoluted mass spectrum of the apo-*ras* with molecular mass 18 854 Da.

(858 Da), indicating the complete denaturation or unfolding of the *ras* protein (data not shown here).

When the pH was adjusted to 5.2 (1 μl of 20 μM *ras*–GDP in 300 μl of 2 mM ammonium acetate), we observed (Fig. 2) a set of multiply-charged ions with the most abundant masses at *m/z* 2413, 2144.9 and 1930.6, corresponding to charge states +8, +9 and +10, respectively. Careful examination of these signals indicated that each peak was actually a cluster of ions. Upon deconvolution of this spectrum (*m/z* 2413, 2144.9 and 1930.6), an average molecular mass of 19 295.6 Da was obtained (inset), which is in good agreement with the theoretical average molecular mass (19 296 Da) for the *ras*–GDP complex. This species was confirmed by a series of ions that resulted from the successive addition of sodium to the *ras*–GDP complex. It should be noted that the *ras*–GDP sample used for this particular experiment had not undergone purification to remove

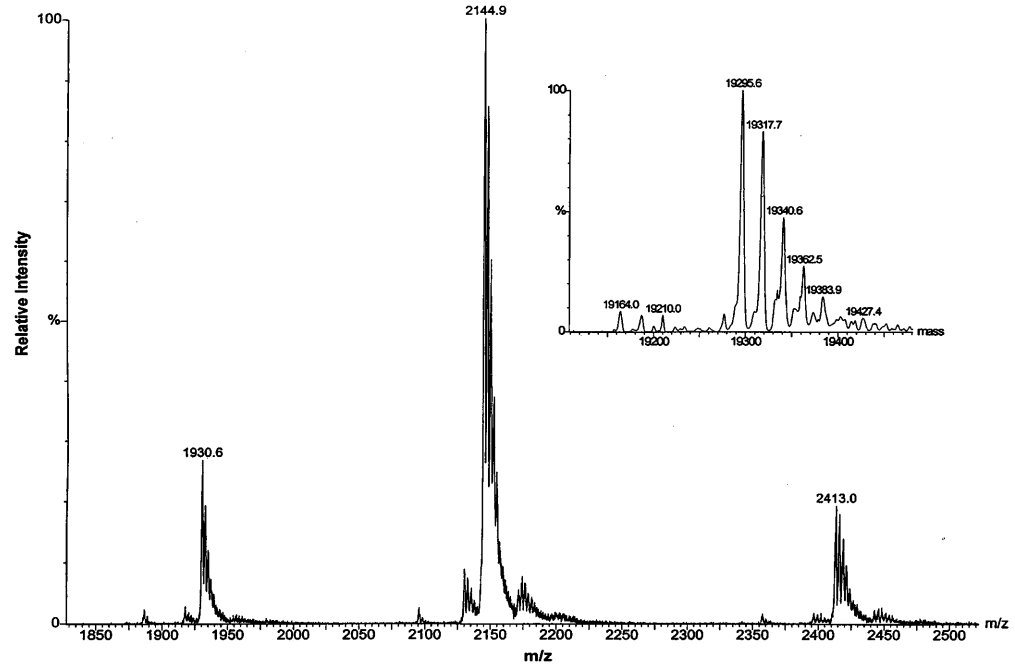

Figure 2. ESI mass spectrum of the *ras*–GDP complex obtained at pH 5.2 in 2 mM ammonium acetate buffer using 20 μM protein solution. Inset shows the deconvoluted mass spectrum of the *ras*–GDP complex with measured molecular mass 19 295.6 Da. Additional peaks in the spectrum correspond to the sodium ion adducts.

sodium salts. In earlier studies,[17,18] protein samples had all undergone this additional purification. The above results demonstrate that the ESI method can tolerate the presence of some salts.

When the ESI experiment was performed on the above sample at pH 4.7 (100 μl of 2 mM ammonium acetate containing 6 μl of 0.1% acetic acid), the mass spectrum shown in Fig. 3 was produced. The distribution of ions has been broadened and is now centered at the +11 charge state with signals at *m/z* 2144.5, 1930.5, 1755.5, 1609.0 and 1488.5. This broadening of charge distribution reflects a small conformational change in the protein making more basic residues available for protonation. The deconvoluted spectrum provided an average molecular mass of 19 294 Da, which is again in good agreement with the theoretical value of 19 295 Da for the *ras*–GDP complex. Thus, despite being partially unfolded, the *ras*–GDP non-covalent complex is still intact.

When the pH of the *ras*–GDP solution was adjusted to 3, the ESI mass spectrum shown in Fig. 4 was obtained. There are two sets of ion distributions in this spectrum: one set with peaks at *m/z* 2144.8, 1930.4 and 1755 and the other at *m/z* 1885.8–857.8. Deconvolution of the first set of ions provided an average molecular mass of 19 294.4 Da, which corresponded to the intact complex. The second set of ions deconvoluted to an average molcular mass of 18 851.4 Da, which is consistent with the molecular mass of the free apo-*ras* protein. This second bell-shaped distribution of ions is much broader and has been shifted to even lower *m/z* values, which is indicative of a higher degree of protonation with substantial unfolding of the protein. It should be noted that the ESI mass spectrum obtained at low pH values (2–3) does not produce sodiated ions. In summary, with pH values between 4 and 6, the *ras*–GDP non-covalent complex is desorbed as multiply-charged ions into the gas phase, maintaining a structure close to its native solution conformation. However, at pH values of 4.7–4, partial unfolding of the protein is observed with no decomposition of the complex. A further decrease in the pH to 2.8 to 2 results in unfolding of the protein and complete release of GDP. These results are in complete agreement with the data obtained for solution-state NMR (Ref. 25; personal communication by the authors with Y.-S. Wang at Schering-Plough Research Institute, Kenilworth, NJ, USA).

The addition of an organic solvent to the aqueous solution of a protein disrupts its hydrophobic interior pocket, leading to a more extended protein conformation. The behavior of the *ras*–GDP complex upon addition of methanol (or acetonitrile) is a good example of this effect; the addition of an organic solvent causes the unfolding of the protein, which leads to the destruction of the complex. Figure 5 shows the ESI mass spectrum of the *ras*–GDP complex obtained in 20% methanol at pH 5.8 (2 mM ammonium acetate aqueous solution). Even at this high pH, the methanol caused the denaturation of the *ras* protein with a substantial dissociation of the complex; molecular masses were 19 294 Da for the complex and 18 851 for the free apo-*ras* protein. Increasing the methanol content to 50% resulted in the total destruction of the non-covalent *ras*–GDP complex.

Drug–protein–ligand interaction

Because of our considerable success in the use of ESI-MS for the analysis of non-covalent complexes, we decided to apply this methodology for screening potential inhibitors of *ras* protein;[21,26] these are small compounds that bind to the *ras* protein and deactivate *ras*

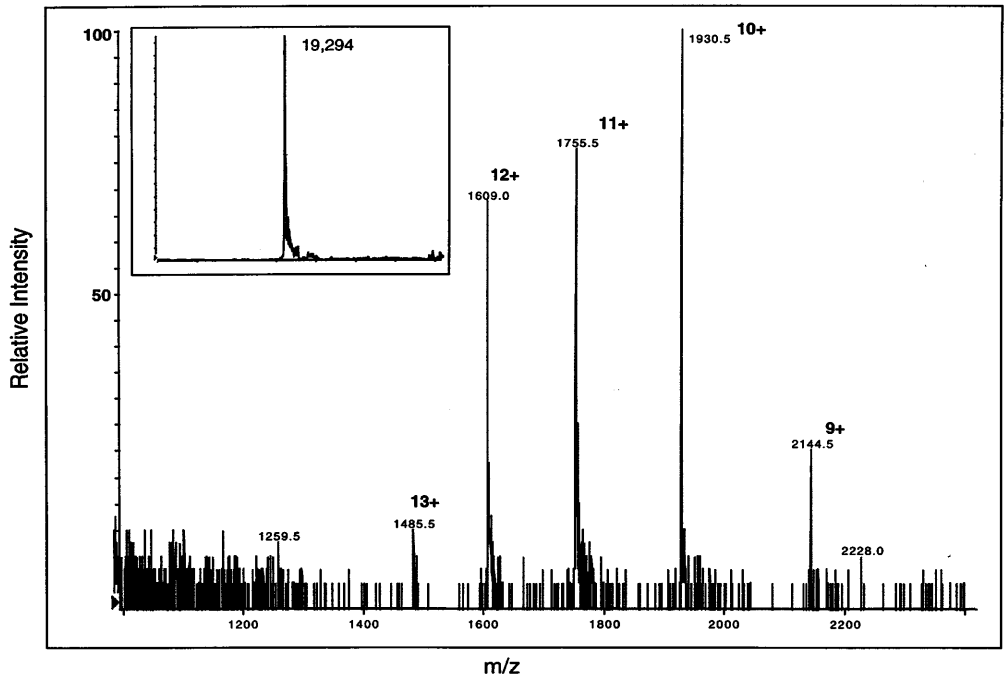

Figure 3. ESI mass spectrum of the *ras*-GDP complex at pH 4.7 in 2 mM ammonium acetate buffer. Inset shows the deconvoluted mass spectrum of the *ras*–GDP complex with molecular mass 19 294 Da.

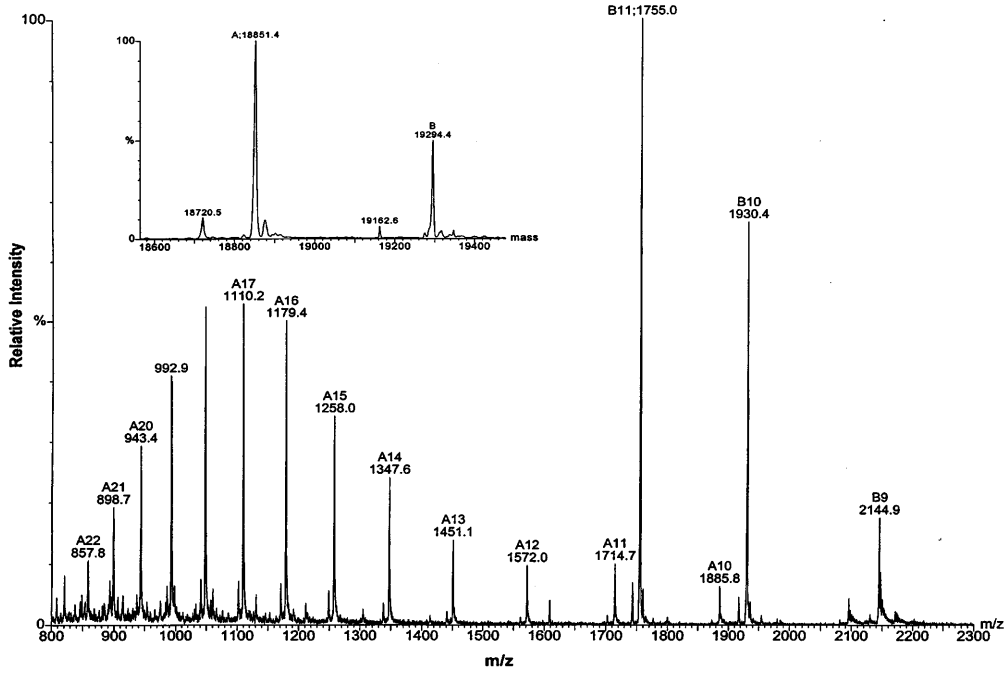

Figure 4. ESI mass spectrum of the *ras*–GDP complex obtained at pH 3 in 2 mM ammonium acetate buffer. Inset shows the deconvoluted mass spectrum of the *ras*–GDP complex with molecular mass 19 924.4 Da and apo-*ras* with molecular mass 18 851.4 Da.

by preventing the exchange of GTP for GDP. The ESI analysis of a solution containing *ras*–GDP and SCH 54292 (2 μg ml^{-1} of complex in 2 mM ammonium acetate at pH 5.8) provided weak, multiply-charged signals with an average molecular mass of 19 816 Da [Fig. 6(A)] representing a ternary non-covalent complex with a 1:1 stoichiometry for *ras*–GDP–SCH 54292. Similar ESI results were obtained for SCH 54341 and an average molecular mass of 19 570 Da was observed [Fig. 6(B)]; however, SCH 53721, which was found to be inactive in biological assays, showed no sign of complexation [Fig. 6(C)]. The *in vitro* activities (IC_{50}) of SCH 54292 and SCH 54341 were 1 and 50 μM, respectively. The fact that SCH 54292 had a very low solubility under the conditions used for our ESI-MS experiment could possibly have contributed to the lower molecular ion abundance observed in Fig. 6(A). The stability of these complexes was highly dependent on pH and the solvent system. Dissociation of the complexes can be brought about by lowering the pH to

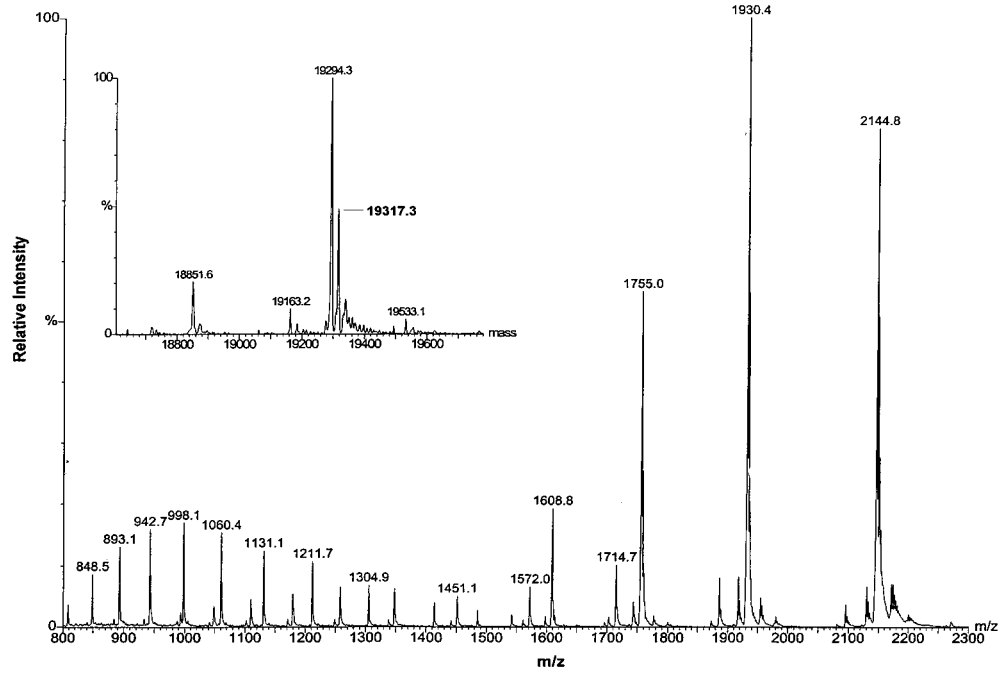

Figure 5. ESI mass spectrum of the *ras*–GDP complex with 20% methanol in 2 mM ammonium acetate buffer at pH 5.8. Inset shows the deconvoluted spectrum with molecular masses 18 851.6 Da (apo-*ras*), 12 294.3 Da (*ras*–GDP) and 19 317.3 Da (*ras*-GDP–Na).

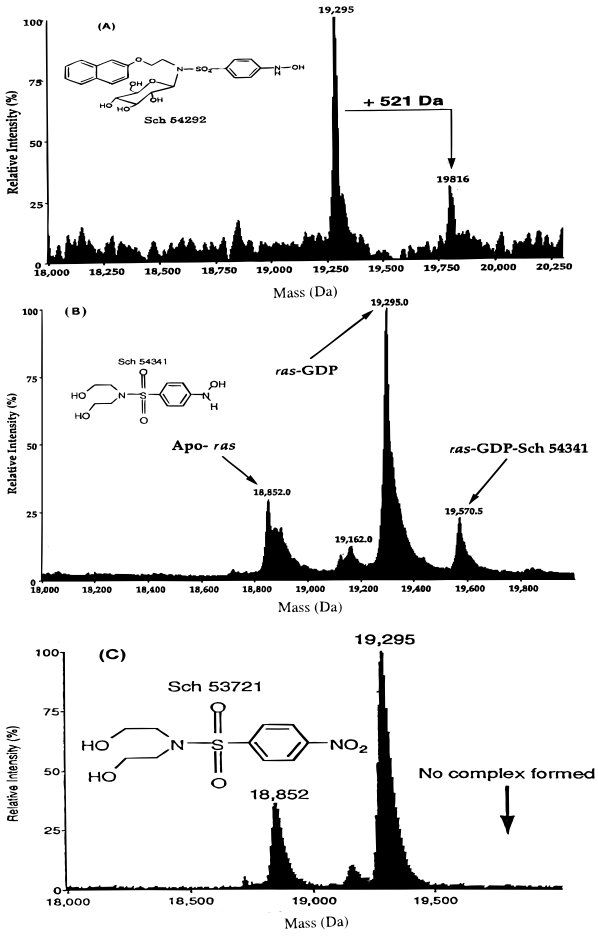

Figure 6. Deconvoluted electrospray mass spectra of (A) *ras*–GDP–SCH 54292 complex, (B) *ras*–GDP–SCH 54341 complex and (C) *ras*–GDP–SCH 53721 complex. In each case the aqueous solution of the complex (2 μg ml^{-1} in 2 mM NH_4OAc buffer, pH 5.8) was infused at 3 μl min^{-1} through the ionspray interface. Several scans (10–20) from *m/z* 300 to 2400 at a scan rate of 2 s per scan were summed to yield the final profile spectrum prior to deconvolution. Reproduced with permission from Ref. 21.

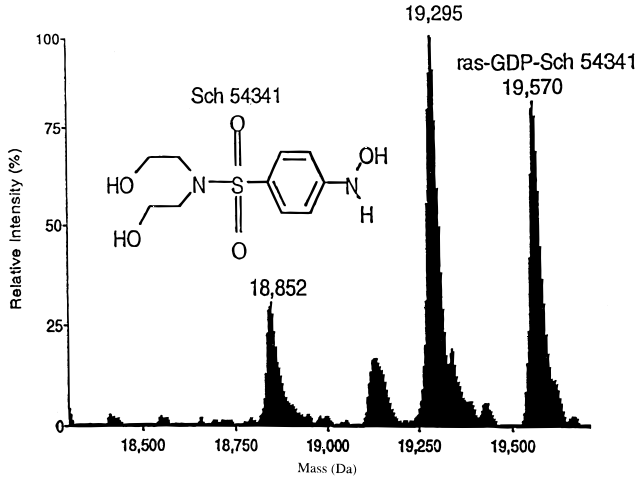

Figure 7. Deconvoluted ESI mass spectrum of *ras*–GDP–SCH 54341 non-covalent complex under refined experimental conditions.

~3.5 or by the addition of 5–10% methanol. Based on the information obtained from our ESI-MS studies with SCH 54292, a large number of related small molecules were synthesized with various functional groups in an effort to enhance the solubility and the biological activity of the drug candidates.

Signal enhancement for these weakly bound complexes was accomplished by refining the experimental protocol by the reduction of parameters that we felt would lower the stress placed on the binding forces in these complexes. Specifically, the infusion flow-rate was reduced to 3 from 10 μl min^{-1}, the orifice voltage was lowered from 80 to 30 V and the nebulizing gas pressure was also reduced. This produced an enhancement of the signal by a factor of 3 (Fig. 7). This enhanced methodology was then applied to screen over 50 inhibitors of *ras*–GDP.

The following strategy was used to gain an insight into the binding location of SCH 54292 on the *ras* protein. Under appropriate conditions, succinic anhydride reacts (covalent modification) specifically with the ε-amino groups of exposed lysine (Lys) residues on the surface of the *ras* protein. It was reasoned that the Lys involved in SCH 54292 binding, or adjacent to its binding site, may be strictly hindered by the bound drug and thereby protected from succinylation. Enzymatic digestion of the succinylated protein followed by LC/MS analysis of the generated peptide fragments provided identification of the unmodified Lys residues. Comparison of the succinate labeling patterns for apo-*ras*, *ras*–GDP and *ras*–GDP–SCH 54292 would indicate which Lys residues were involved in the binding of SCH 54292. With the above approach, it was established that SCH 54292 was bound to *ras*–GDP near Lys-101 without displacing the nucleotide. It is well documented by NMR[27] and x-ray crystallographic studies that GDP is non-covalently bound to the ras protein at Lys-16, Lys-117 and Lys-147 residues.[28]

Protein–protein interaction

Human γ-IFN (interferon) is obtained in a pure form as the result of DNA technology. Mature γ-IFN is a protein consisting of 143 amino acids (molecular mass 16 807.4 Da) with an excess of basic residues. The active form of this protein is a homodimer (molecular mass 33 815 Da) with a K_D value of 50 nM. The detection of the γ-IFN homodimer (a complex composed of identical subunits) by ESI-MS[18] presented many special problems. In principle, the multiple charging phenomenon occurring in ESI can have a strong denaturing effect through simple charge repulsions on the higher order interactions responsible for protein–protein non-covalent association. However, by special sample handling, the γ-IFN homodimer can be preserved in the gas phase. The γ-IFN dimer was observed at an average molecular mass of 33 819 Da in an aqueous solution in which the pH had been adjusted to ~9 by the addition of 30% ammonia solution; a pH of 6.7 was insufficient to observe this dimer. The formation of multiply charged positive ions in a basic solution has been reported earlier for other proteins, myoglobin, lysozyme and lactalbumin.[29] Analysis of the spectrum of a homodimer also requires special attention, since the *m/z* values for the monomer will overlap with the

even-charge states of the dimer. Hence the key to the determination of the presence of the dimer was the detection of the odd-charge state ions: in the case of γ-IFN, a bell-shaped distribution of ions was observed with charge states extending from +15 to +30 (Fig. 8), with the odd-charge states, denoted by an asterisk, indicating the presence of the dimer.[30]

Dilution experiments are necessary to eliminate the possibility that the observed dimer is the result of random protein aggregation that occurs at high protein concentration; protein aggregations will disappear upon dilution whereas a true non-covalent dimer will remain. The protein cytochrome *c* was used for comparison because it is known to form non-specific aggregates. A mixture of an equimolar concentrations of γ-IFN and cytochrome *c* (35 μM) were analyzed by ESI-MS and only the γ-IFN dimer was detected. However, at a concentration of ~0.4 mM very weak peaks corresponding to the cytochrome *c* dimer were detected.[19]

The effect of orifice potential on the stability of the γ-IFN dimer was studied. At potentials above 50 V, the dissociation of the complex begins to occur and at 70 V [Fig. 8(b)] the dimer signals decreased dramatically and virtually no signals corresponding to γ-IFN dimer remained [Fig. 8(c)].

Hepatitis C viruses (HCV)–metal complex

HCV and hepatitis A and B are the main etiological hepatitis agents. The HCV protein has two major domains: the protease domain (HCV NS3) encompassing residues 1–181 of the *N*-terminus portion and the helicase portion that comprises the 450 residues of the *C*-terminus. The HCV NS3 protease domain is tetrahedrally coordinated via three cysteines and one histidine to a zinc ion that stabilizes its tertiary structure. ESI-MS was employed to detect this HCV protease–Zn complex. Figure 9(A) and (B) show this complex at pH 3 and 7, respectively; and an orifice potential of 100 V. The deconvoluted mass spectrum obtained at pH 7 [Fig. 9(B)] indicates an average molecular mass of 21 141 Da, which is consistent with the intact complex, while the spectrum at pH 3 [Fig. 9(A)] corresponds to the free protease domain (molecular mass 21 076 Da). The binding strength of this Zn complex has been extensively studied in our laboratory by varying the orifice potential and with the addition of organic solvents. We have found that this HCV–Zn complex is relatively strong compared with *ras*–GDP, since neither increasing the orifice potential to as high as 160 V at pH 7 nor using a 50% acetonitrile solution (pH 7) had any effect on its stability [31].

Effects of altering orifice voltage

As one would expect, the stronger the binding energy of the non-covalent complex, the less is the effect on the stability of the complex produced by increasing the orifice potential. Increasing the orifice potential increases the internal energy imparted to the complex during the desolvation process and could induce dissociation of the complex. Both *ras*–GDP and HCV–Zn interactions are relatively strong and are little affected by raising the orifice voltage. The HCV–Zn complex is unaffected by an increase in the orifice potential to 160 V (the standard protocol voltage level is 70 V), while *ras*–GDP begins to dissociate at 120 V. In contrast, the

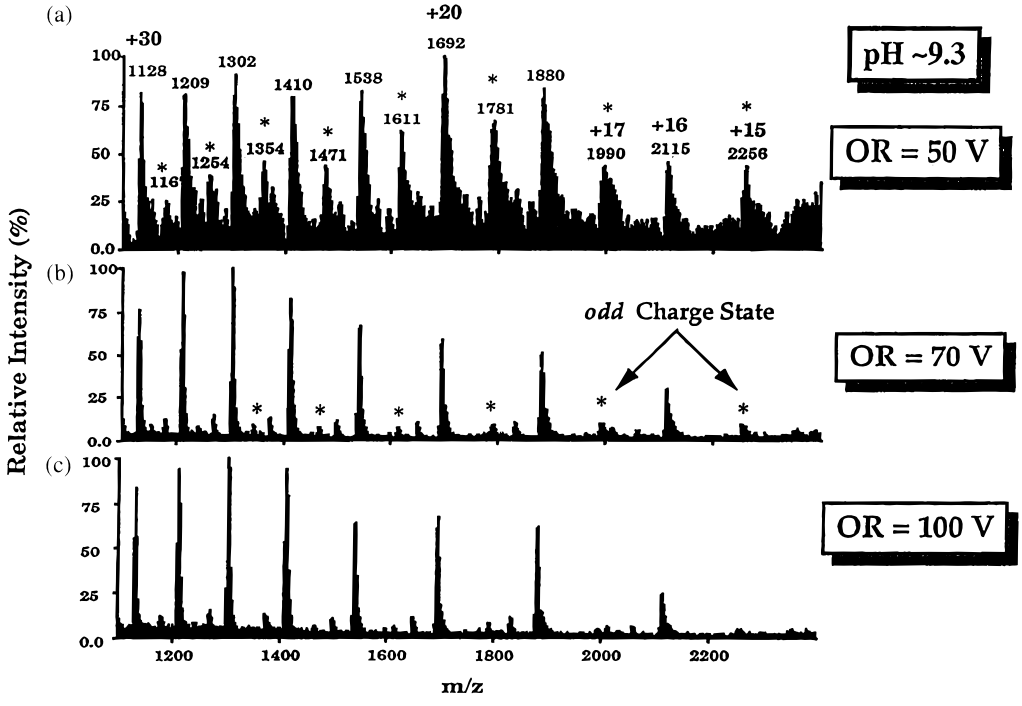

Figure 8. ESI mass spectra of the γ-IFN dimer non-covalent complex obtained from aqueous solution (pH ≈ 9). (a) At orifice voltage (OR) = 50 V, the γ-IFN dimer was detected by the observation of the corresponding multiply charged ions with charge states of +15 to +30; (b) at OR = 70 V, the γ-IFN dimer signals have decreased dramatically; (c) at OR = 100 V, the γ-IFN dimer has almost disappeared from the spectrum. For ease of comparison, the odd-charge states are denoted with an asterisk. Reproduced with permission from Ref. 19.

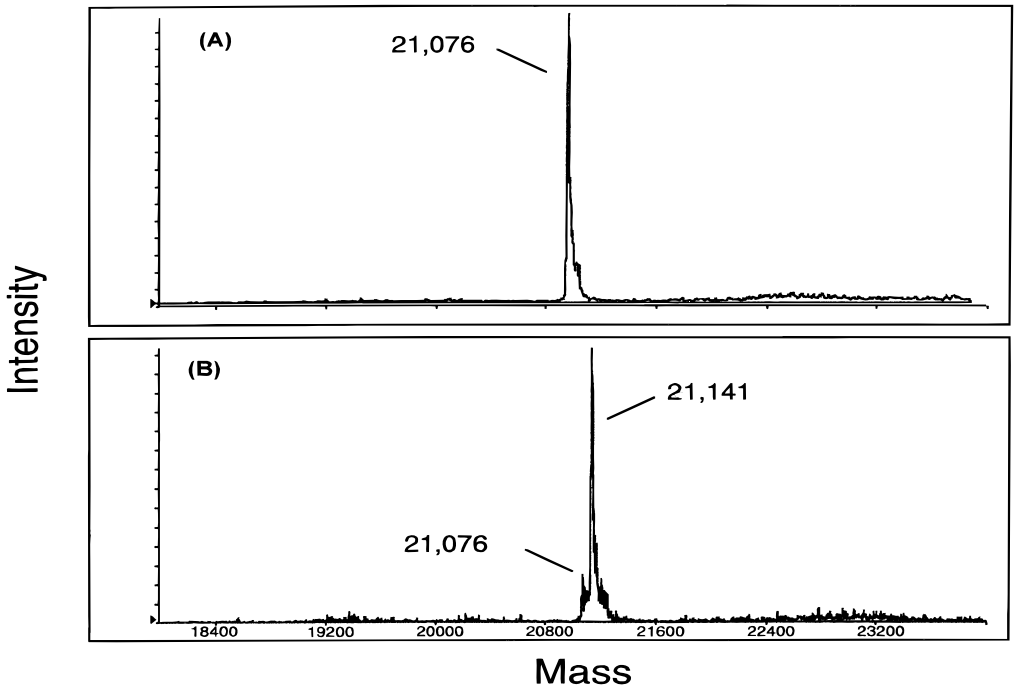

Figure 9. Deconvoluted mass spectra of HCV protease domain (residues 1–181). (A) Mass spectrum obtained at pH 3 and OR = 100 V; (B) mass pectrum obtained at pH 7 and OR = 100 V.

weakly bound γ-IFN dimer started to dissociate at 60 V with complete dissociation at 100 V.

Solution and gas-phase specificity of non-covalent complexes

In non-covalent interactions, the question of specificity of binding is an important issue and one therefore needs to distinguish between actual non-covalent complexes that bind at specific sites and non-specific complexes formed as a result of aggregations (random) due to concentration effects.[19,32,33] The structure of *ras*–GDP has been well estalished by NMR spectroscopy[27] and x-ray crystallography[28] and the binding of GDP to the *ras* protein was found to be highly specific and involves the Lys-16, Lys-117 and Lys-147 residues. The ESI-MS data, discussed above, indicate that the *ras*–GDP complex is preserved in the gas phase when ionized from solutions with pH values in the range 4–6. At lower pH values, however, only a weak signal for the intact complex was observed. To rule out the possibility that concentration effects are responsible for the observed *ras*–GDP complex, dilution studies were performed. The concentration of *ras*–GDP solution was adjusted over a wide range, from 20 to 0.625 μM, while maintaining appropriate experimental conditions. At all concentrations, a strong signal for the intact *ras*–GDP complex was observed. However, in comparison, horse heart cytochrome *c* forms small amounts of non-specific dimer only at high concentrations.[19]

In a second approach for demonstrating the specificity of the *ras*–GDP binding, the *ras*–GDP solution (10 μM) at pH 2.3 (completely denatured with total destruction of the complex) was adjusted to pH 6.2 by the addition of ammonia solution. The ESI mass spectrum of this solution indicated that the *ras*–GDP complex had not reformed, demonstrating the irreversibility of this system. This experiment suggests that the actual *ras*–GDP non-covalent complex observed in ESI-MS is specific in nature and not the result of random aggregation due to concentration effects.

CONCLUSION

The purpose of this tutorial review is to demonstrate the utility of ESI-MS technology for the study of non-covalent complexes and to provide a detailed description of the methodologies employed. ESI is a soft ionization method capable of desorbing non-covalent complexes, at close to their native solution state, into the gas phase as multiply charged ions. In this study, a mass accuracy of better than 0.01% was achieved for protein complexes ranging in molecular mass from 19 000 to over 34 000 Da. This high mass accuracy, far superior to the traditional methods of SDS-PAGE and GPC, makes ESI-MS ideal for the determination of the molecular masses of proteins and their non-covalent complexes. An advantage of this method is that the above analyses were performed on picomole to sub-picomole amounts of material. Another advantage is the rapidity with which reliable data can be obtained to address complex analytical problems. Thus, the stoichiometric ratio of the protein to the small molecule can be accurately and quickly determined. Examples of four different protein non-covalent complexes (protein–ligand, inhibitor–protein–ligand, protein–protein and protein–metal complexes) were taken from our published work to illustrate the power of this technique.

This paper has demonstrated that the key to success in the study of non-covalent protein interactions depends on the careful understanding and manipulation of ESI source parameters and sample solution conditions. The following instrumental parameters and solution conditions were found to be important to the progress of the experiment: (i) the orifice potential must be sufficient to ionized the protein complex, but care must be taken to prevent dissociation; the weakly bound γ-IFN homodimer requires 40 V for ionization but begins to dissociate at potentials as low as 60 V; (ii) the acidity of the solution is also critical; even the relatively strong complex *ras*–GDP begins to dissociate below pH 3; and (iii) it was found that the use of an organic cosolvent is strictly prohibited because the hydrophobic interior pocket of the protein would be disrupted, leading to denaturation; even the addition of 10% methanol results in partial dissociation of the *ras*–GDP complex.

This paper also illustrates the usefulness of ESI-MS for addressing biological problems such as the detection of weakly bound inhibitors. This approach can lead to the discovery of more potent therapeutic compounds based on their structure–activity relationships. Hence ESI-MS can be used for the rapid screening of potential drug candidates, which is especially important in the case of combinatorial chemistry programs.

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