New mass spectrometric methods for the study of noncovalent associations of biopolymers

Richard D. Smith, James E. Bruce, Qinyuan Wu and Q. Paula Lei

Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, PO Box 999, Richland, WA 99352, USA

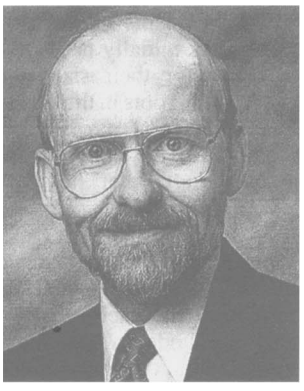
The use of electrospray ionization–mass spectrometry (ESI–MS) for the characterization of noncovalent complexes of biomacromolecules in solution is based upon the gentle nature of the electrospray process that allows a wide range of associations to be transferred intact to the gas phase as fully desolvated complexes. Examples include multimeric proteins, oligonucleotide duplexes, DNA–drug complexes and enzyme-inhibitor complexes. Various studies have indicated that at least some qualities of the three-dimensional solution structures are retained in the gas phase. Recent investigations have also shown the relative stabilities of complexes in the gas phase can be very different than the same complexes in solution. In spite of this, the use of very gentle electrospray interface conditions can provide a direct reflection of relative solution abundances for similar complexes. Competitive binding experiments using sets of ligands have been shown to yield insights regarding relative binding affinities in solution. The potential for high throughput affinity screening of combinatorial libraries using ESI–MS is described based upon the multi-stage MS capability of Fourier transform ion cyclotron resonance instrumentation and involving the characterization of components (after dissociation) of the library constituents initially present as complexes with a target biopolymer in the ion trap. This approach combines, in one rapid experiment, both affinity selection by complex formation with a biopolymer and the identification of the ligands selected from combinatorial mixtures, thus providing information on the relative binding affinities of the library constituents. The present status, limitations and promise of these methods are discussed.

Richard D. Smith is Leader of the Advanced Separations and Mass Spectrometry Group and Director of the FTICR Mass Spectrometry Facility at the Pacific Northwest National Laboratory (PNNL), located in Richland, Washington. His research involves the development and application of nanoscale separations and mass spectrometry to biological studies. Dr Smith is the author or co-author of more than 300 publications, and has been awarded eleven patents and three IR 100 Awards. He is an adjunct faculty member of the Department of Chemistry, Washington State University, and an affiliate faculty member of the Department of Chemistry, University of Idaho.

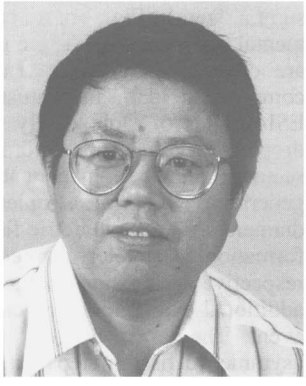
Qinyuan (Quincey) Wu is currently a research scientist in Hoechst Marion Roussel, Inc. Dr Wu received his BSc degree (1982) in physical chemistry from Xiamen (Armoy) University, China, and MS (1990) and PhD (1993) degrees from The University of Illinois (Chicago). Dr Wu's PhD research focused on the interactions of hyperthermal organic ions with metal surfaces. As a postdoctoral fellow at Pacific Northwest National Laboratory, his research involved both fundamental and applied aspects of biological mass spectrometry using electrospray ionization

Q. Paula Lei is currently a postdoctoral fellow at Pacific Northwest National Laboratory. Dr Lei graduated with a PhD in Chemistry from Professor Jon Amster's group at the University of Georgia in 1996. Her research interest focus on bioanalytical mass spectrometry, including non-covalent complexes of biomolecules and mass spectrometry coupled with separation techniques as such capillary electrophoresis.

James E. Bruce joined Dr Smith's group in 1992 after receiving a PhD in Physical Chemistry at the University of Florida and is presently a Senior Research Scientist at PNNL. Dr Bruce's research at the high field FTICR Facility has focused on the development of ion manipulation and detection techniques to enable new biological applications of mass spectrometry, including the direct bioaffinity analysis approach and the analysis of large individual ions using FTICR



Richard D. Smith



Quinyuan (Quincey) Wu

Q. Paula Lei

James E. Bruce

1 Introduction

Structurally specific noncovalent associations of biopolymers dictate a large fraction of biochemical processes, from the expression of genes to the activity of gene products (*i.e.* proteins), and often involve complex and competing pathways where small changes to either the physiological milieu or chemical modifications to the biopolymer (*e.g.* phosphorylation of specific amino acid residues of proteins) can lead to significant biological consequences. Gaining an understanding of the relevant chemical processes and the role of subtle structural modifications is at the heart of much biomedical research, and has practical implications that include the development of gene therapies, new vaccines (*e.g.* to cancer), and new drug leads. The difficulties involved in unravelling the underlying complexities include the generally large size of the participating biopolymers and the practical limitations upon sample quantities available in most cases for *in vitro* studies. The availability of a high resolution structure for a biopolymer (from crystallography or NMR) in many ways only serves to elevate the detail of the chemical questions while providing a scaffold for understanding their interactions. The Human Genome Program will increasingly exacerbate the need for faster and better methods for the study of large biopolymers and their interactions due to the rapid increase in the number of human genes available for study.

It is axiomatic that scientific progress is closely coupled with the development and application of improved analytical techniques. The development of gel electrophoresis, for example, provided a tool now utilized in most biological research, with applications ranging from DNA sequencing to binding assays. Electrophoretic separations in gels are based upon molecular size and shape, and in many ways electrophoresis is analogous to low resolution (time-of-flight) mass analysis in a condensed medium.

In recent years several developments have dramatically extended the utility of mass spectrometry (MS) for the study of large biopolymers, traditionally the domain of electrophoretic methods. Recent research has demonstrated its application for characterization of biopolymers extending to a mass greater than 200 kDa while providing detection limits in the attomole range (and below) and mass measurement precisions that exceed those provided by electrophoresis by more than three orders of magnitude! These advances have largely been derived from the introduction of electrospray ionization (ESI),[1] which has even been demonstrated to allow the ionization of intact viruses[2] and DNA segments of more than 100 MDa.[3] Recent advances in mass analyser technology have also played an important role.

Although a number of details of the ESI process remain a subject of speculation and debate, and there is yet limited information concerning the 3D structural conformation of large ions in the gas phase, it is now incontestable that many weak associations in solution do survive transfer to the gas phase. A substantial literature now demonstrates that a wide range of complexes from solution conditions (that can often mimic those of physiological interest) can survive the ESI process.[4] Examples of noncovalent complexes observed by ESI–MS include enzyme–substrate, receptor–ligand, host–guest, intact multimeric proteins, DNA duplex and quadruplex species, oligonucleotide complexes with drugs and proteins, and protein–drug complexes.

Upon first consideration, the study of solution interactions based upon their analysis in the gas phase appears to be an unlikely approach. In this regard, the distinctively 'gentle' nature of the ESI process is of particular significance. Numerous experimental studies have now indicated that using appropriate ESI interface conditions, compact structures are retained and, at least in some cases, significant structural features either survive or incur only modest changes. Once in vacuum and fully desolvated, the available results indicate that noncovalent complexes can be stable for indefinite periods. The lowest energy structures in the gas phase will almost certainly be different from those in solution, but the magnitude of both the kinetic and thermodynamic constraints upon transitions between these structures for the multiply charged species in the gas phase are presently unknown. There are also reasons that gross structural features might be retained for multiply charged proteins in the gas phase. In solution, the compact protein core structure substantially arises due to hydrophobic interactions and charge sites are most likely to reside on the surface in contact with the aqueous continuous phase. After removal of the dielectric solvent, Coulombic repulsion for the multiply charged protein will be minimized for charge sites on the surface—the number of which depends upon the ESI charge polarity, solution composition, molecular shape and the chemical nature of the charge site in the gas phase. A key question is whether sufficient internal excitation exists in the gas phase to propel conversion of the solution structure to a lower energy (desolvated) structure. The underlying fundamental issue is the role of solvent in maintaining the structural features of biopolymers.

The ability to transfer biopolymers to the gas phase presents new opportunities; not only can measurements be made with incredibly high sensitivity (even a single ion can be repetitively manipulated and accurately weighed[5]) and enormous resolution, but an increasing array of chemical reactions and other processes (*e.g.*, H/D exchange[6]) can also be invoked. Before we discuss the experimental methods and the potential scope of their application, we first outline our view of the electrospray process and how it allows noncovalent complexes to be transferred to vacuum and desolvated while retaining important structural aspects.

2 The electrospray ionization process

Electrosprays are typically produced at near ambient pressure from a liquid stream emanating from a capillary in a strong electric field. The electrostatic nebulization process produces an average droplet size that increases with flow rate, and is in the micron size range for low $\mu l\ min^{-1}$ flow rates. Droplets carry a large net positive or negative charge (depending upon the applied voltage gradient), allowing either positive or negative ions to be produced. The electrospray is directed towards an aperture or capillary inlet, allowing charged particles to be transferred through differentially pumped regions to the mass analyser. During this transfer, heat (particularly at higher pressures) or electric fields (at lower pressures) are the primary variables that determine the species detected by the mass spectrometer.

Perhaps the most widely accepted model for formation of small ions by electrospray is the ion evaporation mechanism.[7] In this model, charged species desorb from the highly charged droplet surface due to the locally high electric field. This process remains speculative for larger ions (*e.g.* biopolymers).

We believe an alternative mechanism of ion formation summarized in Fig. 1 more satisfactorily explains key experimental observations.[4] The charged droplets initially produced are close to the charge (Raleigh) limit for their size and composition. In this mechanism, which has its roots in the initial ESI studies of Dole nearly three decades ago,[8] asymmetric droplet fission occurs as droplets shrink to the point where surface tension is inadequate to maintain droplet stability, generating offspring droplets having significantly smaller diameters. The asymmetric fission process facilitates the rapid formation of smaller charged droplets that are close to their respective maximum charge, and only a small amount of additional evaporation can cause a second asymmetric fission event. The larger progeny droplets can undergo additional asymmetric fission steps as solvent evaporation again drives them to instability, but the process will be faster and require less energy input for the smaller droplets. Through one or more such

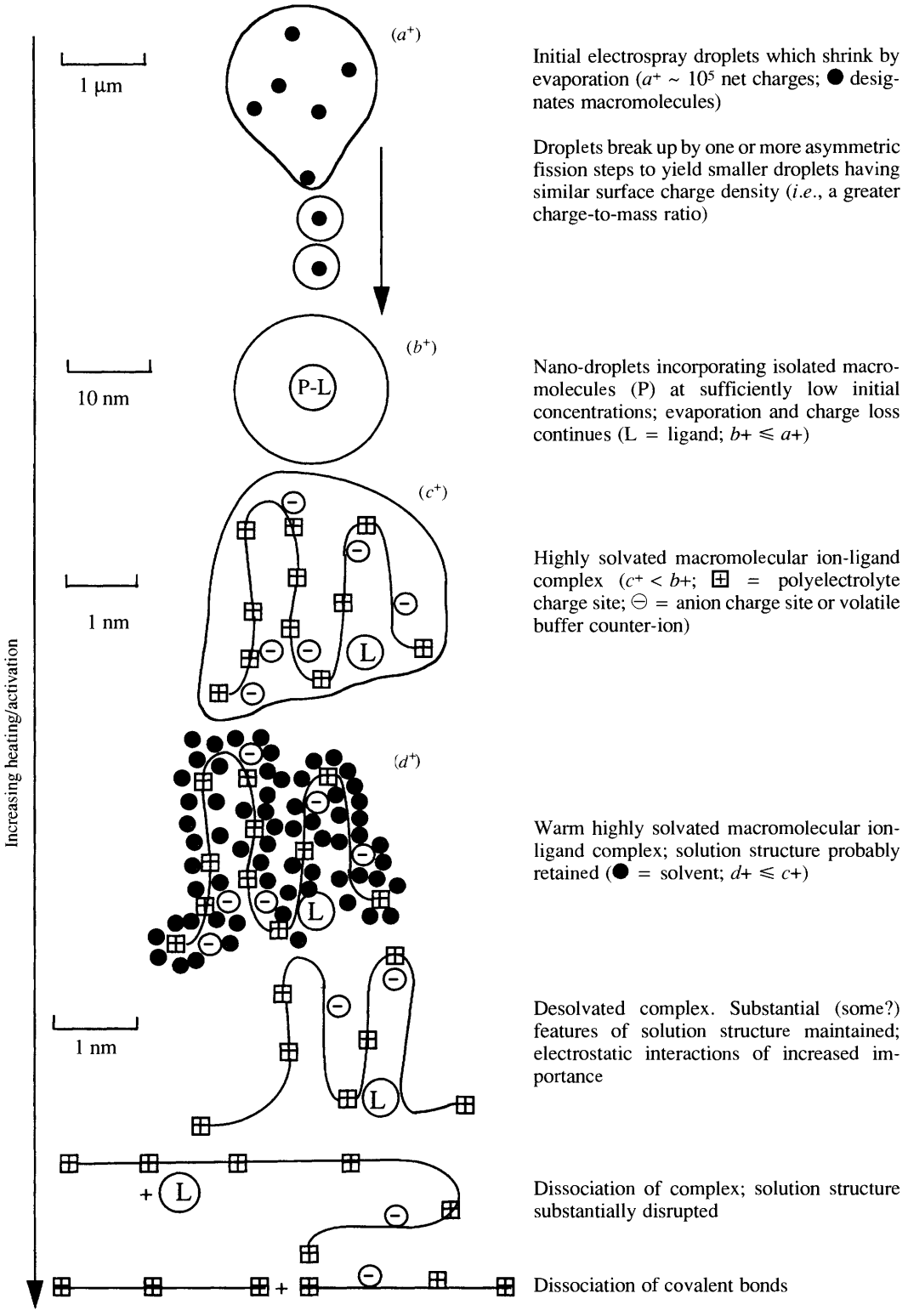


Fig. 1 Model for formation of macromolecular ions during electrospray ionization, based upon asymmetric droplet fission and the resulting formation of small nanometre diameter droplets. With increasing activation, residual solvent and charge are shed. The loss of higher order structure and noncovalent associations (*e.g.* ligand, L) is primarily driven by the extent of activation and repulsive Coulombic forces (after removal of the solvent).

steps it is feasible to isolate single molecules given the concentrations relevant to most electrospray applications. For example, a 10^{-5} M solution initially contains approximately three analyte molecules per 100 nm droplet; a 10^{-6} M solution contains on average only about 0.3 analyte molecules (before any solvent loss by evaporation). Asymmetric fission steps might continue to droplet sizes approaching the dimensions of large proteins (*ca.* 10 nm), and further evaporation yields the molecular ions detected ultimately. The key experimental variable for ESI is the extent to which the charged droplets are heated and the smaller charged species activated by collisions. Sufficient energy may be imparted to just barely cause desolvation or additional activation may be imparted to induce extensive dissociation in the gas phase.

Initially, it was thought that since protein ions were generated by ESI with substantial net charge, their more compact structure

in solution 'stretched out' in the absence of solvent due to Coulombic repulsion between charge sites. It gradually became evident that essentially the inverse is probably true; electrosprayed ions are more highly charged because they are denatured and in a form readily extended prior to ESI (or to a lesser extent, when greater heating occurs during ESI).[9] Lower charge states are produced from more compact structures.[4,10] Although additional more closely spaced charge sites give rise to greater long-range repulsive contributions, the energy differences that occur over distances typical of chemical bonds, and available to drive dissociation, are quite small. Thus, although the activation barrier for breaking a weak bond may be lowered due to the increased Coulombic contributions, noncovalent associations based upon multiple weak electrostatic, hydrogen bonding and dispersive interactions can apparently survive in the absence of excessive thermal activation to the molecular ion. (A corollary is that once the gas phase structure is disrupted, and charge sites are moved apart, it is implausible that molecules will refold unless charging is reduced, and even then it is extremely unlikely that they would refold to the same 'native' structure as in solution.) As charging increases for a given molecular structure, the 'effective' proton affinities of charge sites for positively charged proteins (the basic amino acid residues), for example, decrease to a level at which proton transfer to volatilized solvent in the gas phase can occur.[11] Thus, this model implicitly predicts that the charge state distribution will depend upon both the detailed three-dimensional molecular structure and the location (and nature) of charge sites for the multiply charged macromolecular ions, consistent with experimental observations.[4]

The desolvation of electrosprayed ions is typically completed in *ca.* 1 ms, and the resulting mass spectra are also strongly influenced by the extent of collisional activation and dissociation in the ESI–MS interface. The loss of noncovalent associations during ESI may be kinetically constrained, allowing noncovalently associated species to remain associated as residual solvent is removed. The likelihood of this will depend upon the location and number of charges remaining on each of the associated species (*i.e.* due to Coulombic considerations), the solvent's role in the noncovalent association, the relative strength and number of attractive interactions between complex constituents compared to solute–solvent interactions, and the extent of complex activation in the interface. Given this complexity, it is not possible at this time to make blanket statements from fundamental considerations; at present, experiment is our best guide.

3 Experimental considerations and mass spectrometric methodologies

3.1 Electrospray source and interface conditions for observation of noncovalent associations

The detection of noncovalent complexes from solution is, of course, of only limited interest unless it can be effectively applied to 'unknown' complexes, *e.g.* where stoichiometry and/or relative binding affinity is not established. The ESI–interface conditions necessary to preserve noncovalent complexes are generally more 'gentle' than those conventionally employed for ESI–MS, although this is not always the case. The presence of excessive amounts of nonvolatile salts will result in adduction to molecular ions, decreasing sensitivity, and can make molecular mass measurements problematic. The use of 'volatile' buffers, such as ammonium acetate, is preferred, and one must consider the possible effects relative to established 'physiological' buffers. In many cases, however, the restriction to volatile buffers is of little importance, since solution pH, temperature and ionic strength are often the primary factors of concern. Low ESI flow rates (<50 nl min^{-1}) are also advantageous since the initial droplet size is smaller (<200 nm), desolvation is faster, and less heating is required for desolvation.[12]

3.2 Distinguishing specific and nonspecific noncovalent associations in ESI–MS

The simple observation of peaks by ESI–MS indicative of a complex constitutes insufficient evidence upon which to infer a structurally specific interaction in solution. Distinguishing between structurally specific noncovalent interactions arising from solution and nonspecific aggregation in solution (and that might also result from the electrospray process), does appear to be feasible with careful selection of experimental conditions. However, the necessary conditions (*e.g.* low analyte concentrations) may not be achievable with certain instrumentation (due to sensitivity limitations, for example). There are several criteria we have advanced for evaluating whether complexes observed in the gas-phase reflect specific associations in solution:[4]

Stoichiometry. Generally, complexes (such as A·B) should be observed without comparable contributions which might arise from random aggregation at higher solution concentrations (*i.e.* A·A, B·B, A·B_2, A_2·B, A·B_3 , *etc.*) in order to be confidently attributed to a specific association in solution.

Gas phase lability. Complexes due to weak binding forces (*e.g.* H-bonding, hydrophobic interactions, *etc.*) are generally more readily dissociated using more severe interface conditions or in tandem mass spectrometry (MS^n) experiments. However, complexes having strong electrostatic interactions (*e.g.* protein–DNA complexes) may be tenacious in the gas phase, and the cumulative contributions of many weak bonds can make complexes having larger binding domains more stable.[13]

Dissociation due to modification of solution conditions. Changes in solution temperature, pH, the presence or absence of buffer components, the addition of organic solvents, *etc.* may greatly weaken or disrupt specific associations in solution and should produce a corresponding change in the ESI mass spectra.

Sensitivity to structural modifications. Perhaps the most unambiguous demonstration of a specific interaction from solution is obtained when a variant of one of the complex components produces a substantial change in the relative intensity of the complex in the mass spectrum.

These criteria have now been extensively applied to model systems, and many examples convincingly demonstrating the detection of specific solution associations have been reported. At the same time, it is possible to obtain spectra that are not representative of solution. It is important to understand the origin of such observations, how they can be avoided, and the potential limitations to the methodology these observations suggest. At the present time 'false negatives', in which a suspected or expected complex is not observed, must be viewed with caution. Unless a complex of similar size and nature can be readily detected, one cannot be completely confident that either complex stability in the gas phase or some aspect of the interface conditions or instrument design does not prevent detection. On the other hand 'false positives' are readily avoidable by applying proper experimental conditions (*e.g.* low ESI flow rates, low analyte concentrations).

The fact that mass spectra showing the expected solution stoichiometries for a range of biopolymers have been reported,[4] and without contributions from nonspecific or random aggregation, constitutes strong evidence for the feasibility of studying such interactions by ESI–MS. However, in addition to these structurally specific associations in solution, a broad range of 'nonspecific' associations (at undefined or multiple binding sites and of indefinite stoichiometry) can also occur. A class of ubiquitous noncovalent interactions in solution involves water, an essential component of the native structure of most proteins, where numbers of well localized (*i.e.* 'specific' and 'internal') water molecules are essential to protein conformation. Water is likely to be most strongly retained in association with charge

sites in the gas phase, but is also generally quite specific in its association with hydrophilic protein functional groups on the protein surface and may organize into clathrate-like structures around hydrophobic patches in solution. It is possible to retain some water with electrosprayed species using very 'gentle' interface conditions; however, the absence of 'internal' water molecules in typical ESI–MS spectra would appear to support the view that the protein's structure has been 'extended' from its native tertiary structure. In apparent contradiction, it has also been shown that a compact structure can be retained even after the complete removal of all solvent (Fig. 1). The details as to how large biopolymer ions are 'vacuum dried' but can apparently retain substantial 'memory' of their solution structures remain to be determined.

It is of course impossible to prove beyond all doubt that a specific association exists in solution solely on the basis of ESI–MS results. A recent study for several model proteins indicated that aggregates observed by ESI were largely due to 'non-specific' associations already existing in solution,[14] although small contributions due to random associations are often observed for very large solute concentrations. (In some cases, it may be feasible to experimentally distinguish non-specific and specific complexes based upon their gas phase behaviour; see Section 6). Ideally, ESI–MS will be augmented by independent methods, although the extension to less well-characterized systems is increasingly justified as experimental limitations are defined. When appropriate experimental technique is practiced with amenable instrumentation, the sensitivity and speed of ESI–MS methods suggest its utility for effectively resolving questions of relative binding and stoichiometry. Of particular significance is the capability for conducting studies given only extremely small sample sizes (many orders of magnitude less than required for detailed structural studies) and often with the complex or heterogeneous samples that are the norm for larger biopolymers. In this regard recent developments in mass spectrometric instrumentation are of particular significance.

4 The use of advanced methods based upon ESI–Fourier transform ion cyclotron resonance (FTICR)–mass spectrometry

The combination of ESI with the recent rapid advances of FTICR–mass spectrometry is greatly extending capabilities for the study of large biopolymers.[15] Ions are radially confined in the FTICR trap by an applied magnetic field and axially confined by a trapping potential which is applied orthogonal to the magnetic field. The frequency of the cyclotron gyration is inversely proportional to the mass-to-charge ratio (m/z) and directly proportional to the strength of the applied magnetic field.[16] The orbiting ion clouds induce image currents on two or more detection electrodes which, when Fourier transformed, provide an extremely precise measurement of the cyclotron frequencies (and thus the m/z values) and can simultaneously yield both ultra-high resolution and high mass measurement accuracy. This non-destructive FTICR detection scheme is exploited for ion re-measurements of the same ion population and in tandem or multi-stage (*i.e.* MS^n) dissociation experiments from the introduction of a single group of ions that can be dissociated and remeasured multiple times, providing extensive structural information with minimal sample consumption.

The simultaneous combination of extended mass range and unequalled mass measurement accuracy, resolving power, sensitivity, and MS^n capabilities distinguish FTICR. FTICR also affords the capability for selectively accumulating specific species in the ion trap using quadrupole excitation methods.[17] The combination of capillary electrophoresis with ESI–FTICR analysis recently allowed the analysis of proteins from a single red blood cell.[18] The combination of capillary isoelectric focusing with ESI–FTICR analysis of a 2% red blood cell lysate has recently yielded high resolution spectra for carbonic anhydrase without interference from the *ca.* 100-fold excess of haemoglobin.[19] Utilizing broadband quadrupole excitation methods for improved trapping of ion populations and sustained off resonance irradiation (SORI) methods for collisional dissociation of ions in the FTICR ion trap, partial amino acid sequence information could be obtained from the injection of *ca.* 75 erythrocytes into the separation capillary. MS^n measurements allow the determination of partial amino acid sequences (the basis for rapid protein identification as well as localization of the site and nature of post-translational modifications).

The observation of intact noncovalent complexes by ESI–MS presently requires a balance between providing sufficient heating/activation for ion desolvation, and keeping interface conditions mild enough to preserve the complex (Fig. 1). Insufficient desolvation will cause a loss of sensitivity. However, increasing ESI source heating or collisional activation in order to increase sensitivity can induce dissociation of complexes and provide a distorted view of solution associations. Increasing the biopolymer concentration is generally not a satisfactory solution since it will typically increase non-specific aggregation. It is likely advantageous for the final stages of the desolvation process to proceed slowly, something that is most readily accomplished with mass spectrometers based upon ion trapping technology; *i.e.* either the electrodynamic 'quadrupole' ion trap or FTICR instrumentation. Trapping methods allow the use of much gentler electrospray source conditions than generally applied, since the ion population initially trapped in the FTICR can be (on average) significantly solvated. The ion population can then be desolvated on a much longer time scale than possible with conventional instrumentation ($>$ 100 ms for FTICR *vs.* $<$ 100 μs for conventional instrumentation). This approach allows ions to be 'vacuum dried' in the gentlest fashion feasible, does not require 'tuning' of source parameters for each system, and provides higher sensitivity. As illustrated in Fig. 2(*a*), the need for greater activation for desolvation of some ions results in excessive activation and dissociation of others. Qualitatively, this behaviour is rationalized by the dissociation rate *vs.* 1/(temperature) behaviour being much 'steeper' (*i.e.* due to a larger energy requirement) than the desolvation rate behaviour: thus desolvation dominates at low temperatures and long times while

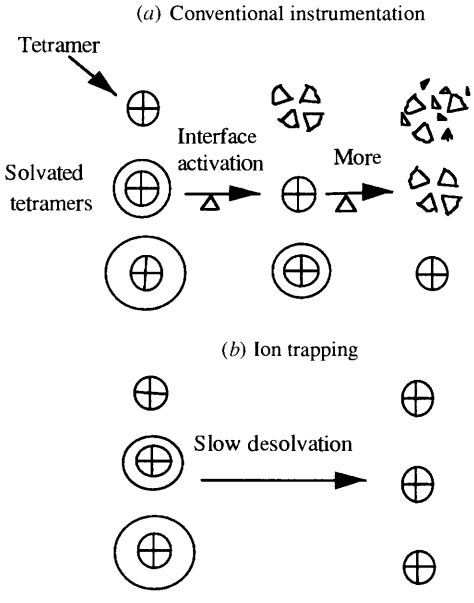


Fig. 2 Comparison of the different results obtained for desolvation due to the different characteristic timescales and necessary heating with conventional and ion trapping mass spectrometers (*e.g.* FTICR). A tetrameric complex is indicated having an initially broad variation in the extent of solvation. A slower desolvation avoids excessive activation in the ESI interface, but results in greatly decreased sensitivity if incomplete at the time of analysis.

dissociation becomes dominant at the higher temperatures needed to drive rapid desolvation. The advantage of this approach is that all trapped ions can be desolvated and that the searching for 'proper' ESI source and interface conditions will be minimized. Available evidence to date indicates that biopolymer complexes are stable in the trap for periods of at least minutes to hours.

5 Examples of noncovalent complexes subject to study

5.1 Multimeric proteins

The intact specific complexes for a number of multimeric proteins have been studied.[10,20,21] Initial studies included such tetrameric proteins as haemoglobin, avidin, streptavidin, the chaperone protein SecB, and concanavalin A. The spectra generally show only a few peaks corresponding to different charge states of the same multimeric species and consistent with the known solution stoichiometry. Peaks indicative of trimeric and possibly pentameric species are not observed, consistent with their origin from specific solution associations. The peaks appear at relatively high *m/z* and have a 'narrow' charge state distribution, observations that have been taken to infer a compact shape and minimal structural heterogeneity.[10] At higher ESI source temperatures the multimeric proteins readily dissociate.

One of the strongest protein–ligand interactions (K_D *ca.* 10^{-15} M) found in nature is that of avidin (a 64 kDa glycoprotein) or streptavidin with biotin, which has wide applicability in biochemistry and molecular biology. Avidin is composed of four identical subunits which associate into the active form. Each of the four subunits can accommodate one biotin molecule. The tetrameric complexes of avidin and streptavidin with biotin have been studied by ESI–MS, and are entirely consistent with this understanding.[22,23] The streptavidin complex with iminobiotin (a biotin analogue with K_D *ca.* 10^{-7} M) was observed to be weaker than the biotin complex, qualitatively consistent with solution behaviour.[23]

5.2 Oligonucleotides and oligonucleotide–drug complexes

It is now well established that double stranded DNA of > 10–12 base pair (bp) size is stable in the gas phase; stability increases with size and larger duplex double-stranded (ds) DNA ions will undergo dissociation of covalent bonds (*e.g.* base loss) in preference to dissociation of the two strands. High resolution ESI–FTICR mass spectra have recently been reported for ds PCR products of > 100 bp size.[24] Noncovalent complexes formed between various drug molecules (both intercalators and minor groove binders) and oligonucleotides have been reported.

5.3 Protein–DNA complexes

Recent work has indicated that complexes of proteins with small oligonucleotides having K_D as low as 10^{-4} M are subject to study by ESI–MS,[25] an observation that likely reflects a significant role of electrostatic interactions. Cheng *et al.* examined the binding of the Gene V protein (a dimeric protein) to a variety of small single-stranded (ss) oligonucleotides by ESI–FTICR.[13] Protein–ssDNA complexes having stoichiometries consistent with known behaviour were observed; only one dimer was found to bind to a 12-mer oligonucleotide, while two dimers were observed as the primary binding motif to the longer 18-mer (*i.e.* a 4 : 1 stoichiometry consistent with the known stoichiometry of one Gene V dimer for each eight bases). The use of competitive binding conditions (*i.e.* molar excesses of two or more oligonucleotides relative to Gene V), provided insights into the sequence specificity of complex formation.[13] These and other observations reflected solution behaviour and indicated that the observed complexes in the gas phase do not arise from non-specific electrostatic interactions (for which little difference would be expected for Gene V interaction with oligonucleotides of different sequence or between ssDNA and dsDNA). Previous studies had suggested that the Gene V protein–DNA complex has a stoichiometry in which each protein monomer binds to 3–5 nucleotides, but were ambiguous as to whether each one of these stoichiometries reflects complexes of specific ratio or an average of stoichiometries. The ESI–FTICR study showed a transition from 2:1 to 4:1 stoichiometry at *ca.* 15 nucleotides. Importantly, it was also demonstrated that collisional dissociation methods could be used to break-up the complex, and then retain the Gene V product in the FTICR ion trap, after which collisional dissociation of the Gene V protein provided substantial structural information on the protein.

Another example involves complexes of the eukaryotic transcription factor, PU.1, with ds DNA.[26] PU.1 binds with high selectivity to the recognition sequence GGA(A/T). Fig. 3(*a*) shows that a solution of the PU.1 protein with an excess of a 17 bp duplex ('wild type') having the recognition sequence gives an FTICR mass spectrum in which the protein–duplex DNA complex of expected stoichiometry dominates. Upon adding PU.1 protein to a mixture of ds oligonucleotides containing both the recognition sequence (wild type) and a 'mutant' lacking the recognition sequence under competitive binding conditions, the protein showed binding with only the wild type [Fig. 3(*b*)]. Repeating the same experiment with a 20-fold excess of the mutant duplex again showed only complex formation with the

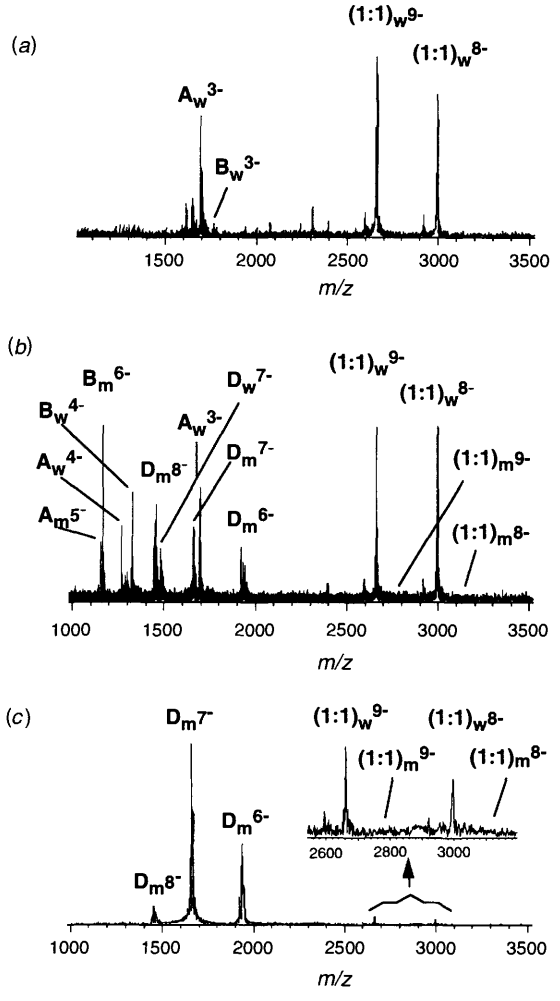


Fig. 3 ESI–FTICR mass spectra showing the complex of the PU.1 protein with a double stranded 17 bp oligonucleotide ('wildtype'; w) having the GGA(A/T) recognition sequence (*a*), and in competitive binding experiments with (*b*) a 1.3 fold and (*c*) a 20 fold excess of a 19 bp oligonucleotide ('mutant'; m) without the recognition sequence. The experiments show the expected 1 : 1 complexes, and excess DNA duplex is evident at low *m/z* as either the duplex (D) or the two individual strands (A and B).

wild type [Fig. 3(*c*)]. Gel shift assays showed that ESI–MS correctly reflected solution behaviour.[26]

6 Can gas phase dissociation studies provide information on binding in solution?

6.1 Experimental insights

Questions related to the observation of noncovalent complexes by ESI–MS include: (1) how closely does the gas phase structure of the complexes resemble that in solution?; (2) can measurement of the stabilities of the gas phase complexes give insights regarding the stabilities of the corresponding complexes in solution?; and (3) if not, how do relative gas phase stabilities differ from those in solution?

To probe these and related questions, we have conducted studies in collaboration with Whitesides and coworkers at Harvard University, using the 29 kDa protein carbonic anhydrase (CA) and its complexes with various substituted benzenesulfonamide inhibitors. CA is a roughly spherical Zn^{II} metalloenzyme having a conical binding pocket, which catalyses the hydration of CO_2 to hydrogencarbonate, and is an attractive model system due to the stability of CA and its well characterized structure and ligand complexes. A large body of data correlates structures of sulfonamide ligands with their binding constants to CA, providing a basis for inferences regarding the protein structure and its ligand interactions in the gas phase.

Fig. 4 shows an ESI–mass spectrum for a mixture of bovine CAII (BCAII) and a set of inhibitors in acidic solution where the native structure of the protein is rapidly denatured. In this case, a relatively conventional ESI–mass spectrum was obtained, even under the gentle interface conditions used, showing a range of charge states extending to $>20^+$. The higher charge states indicate an increasingly disrupted solution structure, which can be attributed to acid catalysed denaturation, and consistent with this the Zn is absent. The lower charge states (at higher *m/z*) show greater retention of Zn, suggesting that *ca.* 20% of the BCAII still retained Zn and a form of intermediate compactness at the time of analysis. Significantly, no complexes with inhibitors were observed, indicating that distinctive binding pocket features were absent in acidic solution (consistent with known solution behaviour) and that random aggregation with inhibitors during ESI does not occur.

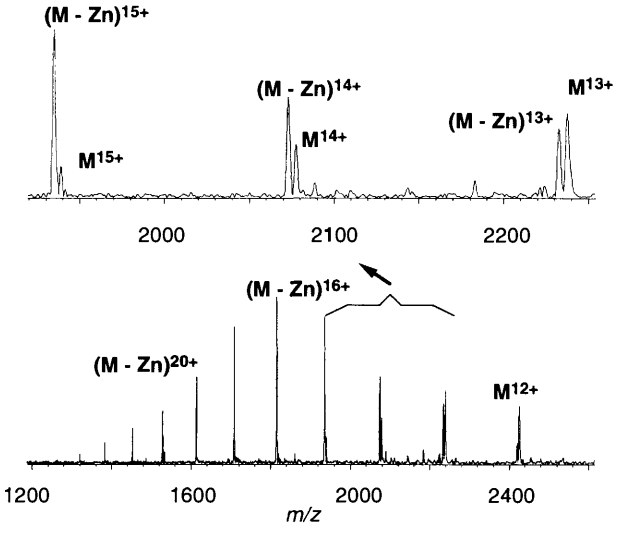


Fig. 4 Mass spectrum of an inhibitor mixture (2.0 μM each) with BCAII (1.0 μM) in 10 mM acetic acid (pH 3.4).[27] No complex ions of inhibitors with BCAII were observed and BCAII has been denatured with substantial loss of Zn^{II}; at lower charge states some Zn retention is evident (top).

A dramatic difference is evident for a similar mixture of BCAII and inhibitors in 10 mM ammonium acetate at pH 7 (Fig. 5).[27] Two lower charge state species (10^+ and 9^+) now dominate the spectrum, indicating the structure is much more compact. A series of peaks is observed corresponding to complexes of BCAII with the various inhibitors, and Zn is fully retained in these gas phase complexes.

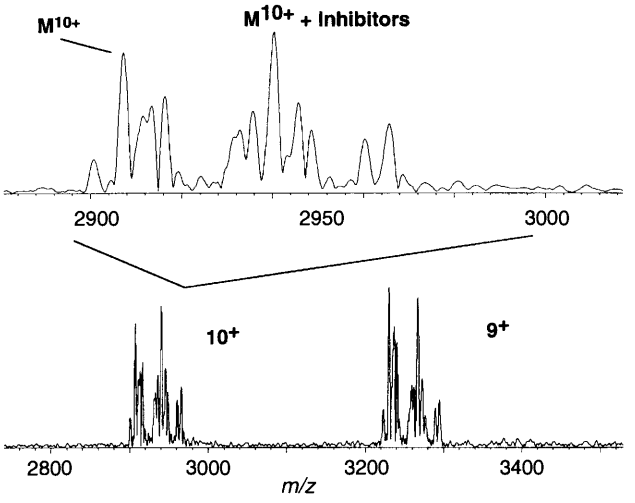


Fig. 5 Positive-ion ESI–FTICR spectrum obtained for a set of 17 inhibitors (0.05 μM each) with BCAII (1.0 μM in 10 mM ammonium acetate at pH 7). The bottom shows the narrow charge state distribution observed, typical of cases where noncovalent associations and a (presumably) compact three-dimensional structure is maintained using gentle ESI conditions. The top spectrum shows detail of the 10^+ charge state region, and shows a series of peaks corresponding to the enzyme–inhibitor complexes and the remaining BCAII.[27]

It was not clear from these results whether the inhibitor remains in the binding pocket after transfer into the gas phase, or whether the binding pocket structure is maintained (*i.e.* although the association of BCAII and inhibitor is maintained, the structure might be altered greatly). Experiments which compared the relative stabilities of BCAII complexes having both one and two identical inhibitors, show that the first inhibitor binds more strongly in the gas phase, consistent with retention in the binding pocket.[28] If structural features are lost, no special gas phase stability would necessarily be expected for the first inhibitor compared to the second. When two inhibitors are associated with the enzyme, at least one of them must be 'non-specifically' bound outside the binding pocket. A difference in the complex stability in the gas phase might be expected, if a binding pocket were substantially preserved in the gas phase and the inhibitor specifically bound in solution remained associated; it is reasonable to expect that an inhibitor in the enzyme binding pocket will be more strongly bound in the gas phase than an identical inhibitor randomly associated on the protein surface. The relative gas phase stabilities of the desolvated bovine carbonic anhydrase II $(BCAII)^{10+}$ complexes with both one inhibitor and two inhibitor molecules (formed using large excesses of inhibitor) have been investigated in the FTICR ion trap using the SORI technique. SORI allows selective collisional heating of species in the FTICR trap. By fixing the frequency difference between the SORI and the complexes' cyclotron frequency, the gas pressure, and the length of the SORI event, the amplitude of the SORI irradiation can be used to vary the extent of collisional activation. Thus, the dissociation efficiency as a function of the SORI peak-to-peak amplitude (V_{pp}) provides a measure of the gas phase stability of the complex. These experiments clearly showed that $(BCAII + 2Inh)^{10+}$ complexes are less stable in the gas phase, with loss of the second inhibitor being significantly more facile, and suggest that the enzyme–inhibitor complexes in the gas phase retain significant features from solution and that the dehydrated binding pocket likely retains the specifically bound inhibitor from solution. Studies also showed that stability is greatly reduced in the gas phase for inhibitor complexes with BCAII–

Zn (*i.e.* the apo-protein) at pH 7 where the structure is known to be largely similar to the intact protein.

Results from other experiments also established that the *para*-NO_2-benzenesulfonamide-human CAII (HCAII) complex is more stable than its *ortho*-substituted counterpart in the gas phase. The bulky NO_2 group in the *ortho* position has a sterically hindered interaction of the inhibitor with the binding pocket and displays reduced binding affinity in solution. In these experiments, the 10^+ charge state ions of the protein–inhibitor complexes were selectively accumulated in the FTICR and then subjected to SORI collisional activation. The HCAII complexes with the *para*-NO_2 inhibitor show greater gas phase stability (Fig. 6). Since solvent does not directly contribute to

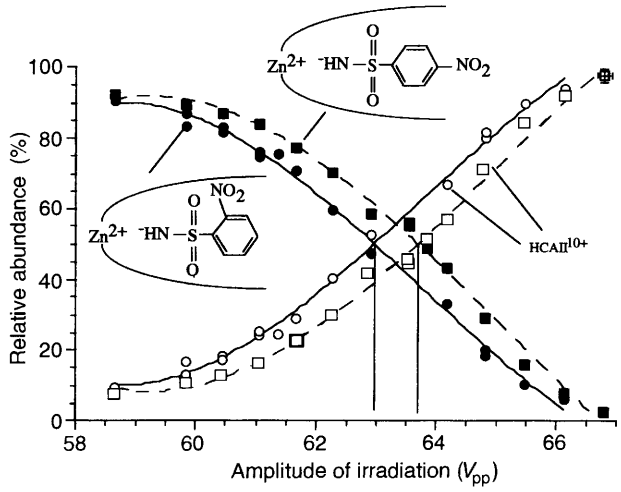


Fig. 6 A plot of the normalized abundances of the *para*- and *ortho*-NO_2-benzenesulfonamide-HCAII complex ions (filled symbols) and the dissociated HCAII product ions (open symbols) *vs.* the extent of SORI activation. The amplitudes of irradiation at the crossing point of the two curves provide measures of the relative gas phase stabilities of the complexes in the gas phase. These results indicate a similar steric effects between the protein and the ligand exists in the gas phase as in solution, and suggest that features of the binding pocket are retained after desolvation in the gas phase.

the steric effect, a similar difference in complex stability in the gas phase would also be expected for these two isomeric inhibitors if the active site structure of the noncovalent complexes were preserved. On the other hand, randomly associated inhibitors (*i.e.* where either the binding pocket no longer exists or the inhibitors do not remain in the pocket after transfer to the gas phase) would not be expected to result in any difference in the gas phase stability. These results also suggest that distinctive features of the binding pocket are retained in the gas phase.

From the experiments described thus far, it appears that carbonic anhydrase can maintain a relatively compact structure in the gas phase, and that the binding pocket retains features that result in stronger inhibitor interaction in the gas phase. A key question is whether measurements of relative stabilities in the gas phase will generally mirror those in solution. Recent studies of BCAII–inhibitor complexes have explored this issue and showed dramatic differences in the relative gas phase stabilities compared with either solution binding constants or solution dissociation rates ('off-rates') for an array of complexes.[29] The gas phase stabilities of BCAII–inhibitor complexes were found to have no direct correlation with the complex stabilities in solution [Fig. 7(*a*)]. A plot of the calculated polar surface area for the inhibitors (that portion of the total molecular surface which is charged, has a large dipole or contributes to hydrogen bonding) *vs.* SORI amplitude revealed good (but different) correlations for sets of inhibitors both with and without aromatic amino acid residues [Fig. 7(*b*)]. The stabilities of the complexes were also found to increase monotonically with the

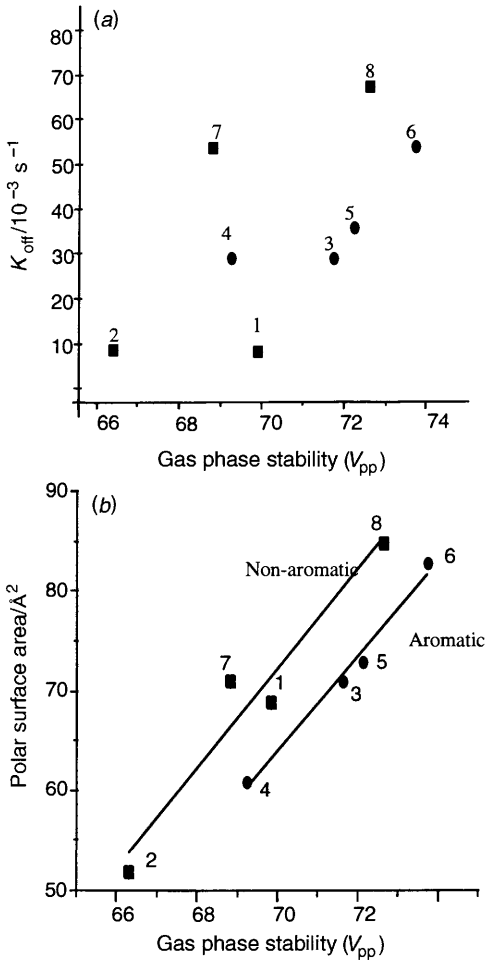


Fig. 7 (*a*) Plot of liquid phase dissociation constant *vs.* gas phase stability for a set of *para*-substituted benzenesulfonamide inhibitor complexes with BCAII measured SORI induced dissociation, showing stability increases with the size of the inhibitor tail.[29] (*b*) Plot of the polar surface areas of the same inhibitors *vs.* gas phase complex stability. The inhibitors having aromatic amino acid residues (circles) show stronger binding with BCAII than inhibitors with aliphatic side chains in the gas phase having the same polar surface area.

number of inhibitor amino acid residues (or the polar surface area). These correlations suggest that the major attractive forces for noncovalent protein–ligand binding in the gas phase are due to interactions between the polar surfaces through electrostatic, dipole–dipole, or hydrogen bonding interactions, and that the inhibitor tail has collapsed to the protein surface in the gas phase, and contributes significantly to complex stability. For inhibitors having the same polar surface area, an aromatic amino acid side chain results in a stronger binding interaction with the protein in the gas phase than does an aliphatic side chain. Thus, whereas the off-rates of BCAII–inhibitor complexes in solution are mainly affected by hydrophobic interactions between the inhibitor and the enzyme, their corresponding gas phase stabilities appear to be primarily determined by polar interactions that reflect the extent of contact area between complex constituents.

6.2 Implications for ESI–MS studies

These results show that attempts to obtain information regarding solution stability directly from the corresponding gas phase measurements will often be problematic. In the case of isomeric inhibitors where differences in solution binding with HCAII are attributed to steric effects, the gas phase results correctly mirrored those in solution, and suggest that the binding pocket retains a crucial role in the gas phase stability. The fact that a

second inhibitor molecule binds with less strength in the gas phase, as does a single inhibitor to the apo protein, adds further support to this view. However, in comparing inhibitors of differing structure, no direct correlation between gas phase and solution stabilities was obtained. This is not surprising given the different environments.

The key implication for applications is that ESI–MS studies should be conducted under the gentlest conditions possible if they are to correctly reflect the relative abundances of the protein–ligand complexes in solution. As we noted earlier, it appears that both the use of low flow rate electrosprays and the slow desolvation (using ion traps) are likely to be advantageous for this purpose. As harsher ESI interface conditions are utilized, the relative abundances of gas phase complexes will be increasingly skewed due to any differences in gas phase stabilities. Thus, studies are more likely to produce misleading results when significant gas phase dissociation of complexes occurs.

Although the evaluation of solution binding based upon gas phase stability is generally to be avoided, this does not prohibit the use of ESI–MS for studies of complexes in solution, but does serve to clarify the experimental requirements (*e.g.* interface conditions affording negligible dissociation of complexes). Studies of small molecule associations that are primarily hydrophobic in nature are most likely to be problematic. The gas phase stabilities of complexes will likely increase as molecular size increases, or more importantly, the available 'contact area' between the constituents increases. Although the generality of these observations remains to be determined, they suggest that ESI–MS may well be broadly applicable to studies involving interactions of larger biopolymers. In the next section, we describe an approach based upon this understanding and the abilities for various ion manipulations provided by FTICR instrumentation, and illustrate its utility for studies involving protein–inhibitor complexes.

7 Application to screening combinatorial libraries

7.1 Bioaffinity characterization mass spectrometry

The traditional approach to drug development has involved the synthesis or isolation of individual compounds followed by their screening and evaluation through a series of chemical and biological assays. An initial step in the screening process often involves identification of candidate compounds that display high binding affinity *in vitro* to the targeted biopolymer. More recently, the selection of pharmacologically active molecules from mixtures, or 'libraries', of compounds has been shown effective and has become a major enterprise. The ability to screen very large libraries to identify candidates for more exhaustive study allows a broad range of molecular diversity to be explored. For example, in the case of tripeptides, a library containing all variations for three amino acid residues will consist of 8000 peptides (twenty possible for each of three residues, *i.e.* 20^3). The number of possible hexapeptides is 6.4×10^7 (*i.e.* 20^6). In the 'combinatorial' approach, many compounds are synthesized (often deliberately producing a complex mixture), and then subsequently examined for their affinity to the targeted biopolymer (or other property), typically by partitioning subsets of the library so as to facilitate the identification of the most active components. This is often performed by binding either the target or library molecules to a solid support medium in a fashion designed to facilitate identification of the active components. The combinatorial approach has proved attractive since it allows much larger numbers of compounds to be generated and screened more rapidly than by serial approaches involving the individual synthesis and assay of each prospective affinant.

The conventional screening of combinatorial libraries is most effective when the synthesis is well behaved (*e.g.* components are generated in nearly equimolar quantities, unsuspected components are not present, *etc.*), the general composition is well established, and suitable analytical methods are available. From this viewpoint libraries consisting of polypeptides or oligonucleotides are attractive, since the well-developed tools of biochemistry and molecular biology may be invoked for their synthesis and analysis. Unfortunately, they also have limited utility as drugs due to their generally rapid degradation by enzymatic processes, and most efforts focus on chemically more diverse libraries where the synthetic and analytical advantages that apply for these linear biopolymers do not currently exist.

We have developed an approach for the screening of combinatorial libraries based upon the capabilities of ESI–MS and the use of advanced FTICR ion manipulation methods.[30] The bioaffinity characterization mass spectrometry (BACMS) approach eliminates the need for distinct separation and/or purification steps involving the original ligand mixture and the problems associated with linking the affinity ligand to a surface since the complex can be formed free in solution at physiologically relevant pH values. The key to this approach is the ability to directly transfer fragile noncovalent complexes from solution by ESI and into the FTICR mass spectrometer.[31] The potential advantages of this approach originate from the ability to first recognize the complex in solution (in which it may have only a very low concentration), to separate it in the gas phase from all other ions (including other complexes), and finally to provide structural information on the complex and/or selected biomolecule. Thus, the separation/affinity selection and analysis steps are combined in one experiment.

A conceptual representation of one implementation of the BACMS approach is given in Fig. 8. The complexes to be investigated are electrosprayed directly from a solution containing both the affinity target and the ligand library, and using

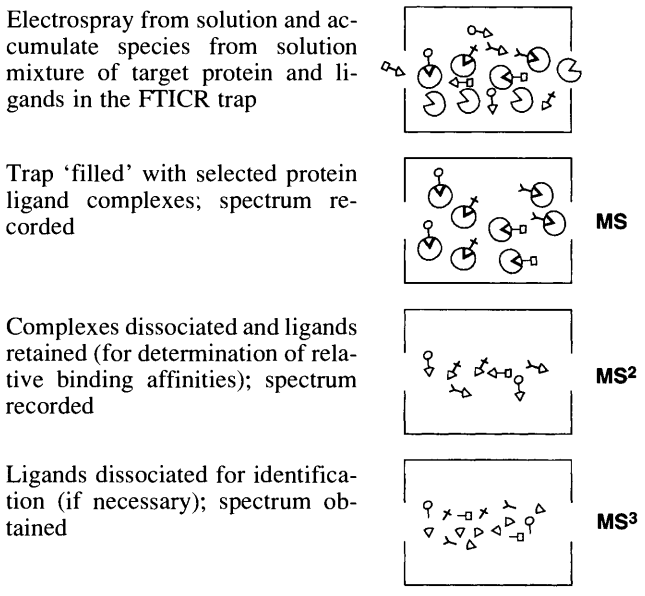


Fig. 8 A conceptual representation of the BACMS technique for the characterization of mixtures of complexes. In this approach the complexes are selectively accumulated in the trap, and then dissociated with retention of the ligands that would then display relative abundances representative of their binding with the targeted biopolymer.[30] Since the ligand ions are retained in the ion trap after a spectrum is obtained, subsequent stages of MS can be used for structural characterization of the ligands by exploiting ion dissociation methods.

appropriate ESI source conditions, transferred to the gas phase and accumulated in the FTICR ion trap. Typically, the complexes of interest are first identified in the mass spectrum and then isolated in a separate step using selected-ion accumulation (SIA) (recent developments allow the selective accumulation of even very low concentration species,[32] which fills the trap to useful capacity of the population of selected ions). This is followed by SORI dissociation of all the

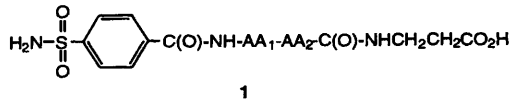
complexes and the retention of the corresponding higher binding affinity ligand species in the trap, after which a high resolution mass spectrum can be obtained. In the same experiment, the inhibitors can be further dissociated to obtain structural information and for definitive identification.[27] A requirement of this approach is that the inhibitor carry a charge. Since the complexes are highly charged, dissociation will generally result in net charges on both dissociation products if charge-carrying sites are available, and either positively or negatively-charged complexes may be selected for this purpose. Alternatively, inhibitor libraries can also be designed to incorporate a charge-carrying site that does not affect relative solution binding.

Since the noncovalent complexes to be investigated with BACMS require that the concentration of the affinity target should be limited to avoid non-specific aggregation during ESI, the complexes of interest may be present only at very low concentrations in solution. Very large libraries would further increase the need for sensitivity, and benefit from the FTICR capability for accumulating trace level constituents (the accumulation of noncovalent complexes from solution concentrations as low as 10^{-9} M has been demonstrated[32]). Importantly, the wide range of FTICR capabilities for high resolution and multi-stage MS analysis are fully available to be applied to species recovered from the dissociation of noncovalent complexes.

7.2 Bioaffinity characterization mass spectrometry of protein–inhibitor complexes

The initial demonstration of the BACMS approach was conducted in collaboration with Whitesides and coworkers. We initially studied two inhibitor mixtures, of 7 and 18 components, that had well characterized BCA–inhibitor binding constants spanning three orders of magnitude (10^{-6} to 10^{-9} M).[27] One group of inhibitors consisted of compounds having dipeptide substituents with one amino acid invariant and the other position incorporating amino acids chosen to be representative of diverse size, shape, hydrophobicity and acid–base properties. The relative abundance ratios of the various complexes observed by FTICR for two series of inhibitors were consistent with their relative affinities towards BCAII in solution.[27] In this work, we also demonstrated the use of multi-stage MS methods for dissociation of the inhibitors to assist their identification (although high resolution analysis was adequate for small libraries).

To demonstrate the extension to larger combinatorial libraries, two mixtures of *para*-substituted benzene sulfonamide inhibitors were synthesized (using solid phase chemistries) (derived from 4-carboxy benzenesulfonamide, **1**), in which all



1

combinations of 17 amino acid residues (AA_1 and AA_2) were incorporated into two positions (17 × 17 = 289 components).[33] (Cys, Met and Trp residues were omitted due to the possibility of oxidation during synthesis.) When a mixture of BCAII (2.5 μM) and a peptide library (0.5 μM for each inhibitor; 289 and 256 compounds for the L- and D-libraries, respectively) in a 10 mM NH_4OAc solution (pH 7.0) was analysed using ESI–FTICR, we observed major peaks in the mass spectra corresponding to intact complexes in the 7^- to 9^- charge states (Fig. 9, top). Note that the individual protein–ligand complexes cannot be resolved due to the large and overlapping isotopic envelopes for the different complexes, a complication that results primarily from the size of BCA. Thus, it is apparent that direct mass spectrometric analysis of the complexes is impractical, regardless of mass spectrometer resolution. On the other hand, collision-induced dissociation of the selectively accumulated 9^- charge state complexes primarily produced the

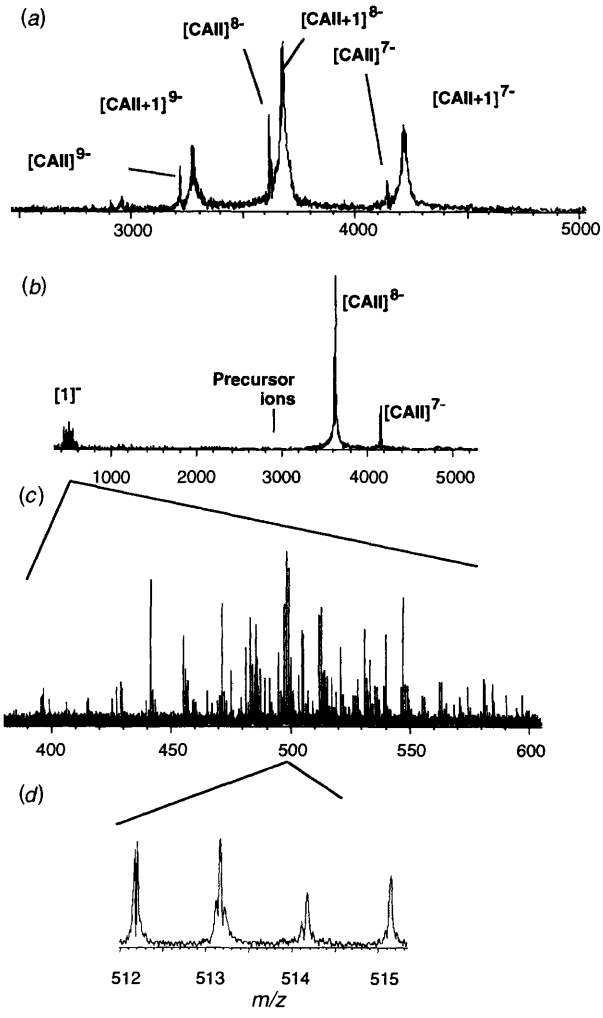


Fig. 9 (*a*) ESI–FTICR mass spectrum from a mixture of the 289-component L-library (0.5 μM each) and CAII (2.5 μM) in 10 mM NH_4OAc (pH 7.0).[33] (*b*) SORI dissociation of the isolated complex ions of $[CAII+1]^{9-}$. The dissociation conditions were set such that the complex ions were completely dissociated. The insets (*c*) and (*d*) show expanded views of the singly charged inhibitors, $[1]^{1-}$.

8^- charge state of the protein and singly charged negative ions for nearly all the inhibitors ($[1]^{1-}$, Fig. 9). The resulting mass spectra allowed the dissociated ligands to be identified based upon their different molecular masses; their ion intensities provide a measure of the relative binding affinities. The correlation is clearly sensitive to the equilibrium concentration of the protein. To obtain quantitative information about binding constants, the equilibrium concentration of the protein must be accurately known, or a correlation curve must be calibrated using internal standards.[33]

This approach has several implicit assumptions. First, there should be minimal or no dissociation of the complexes during ionization, ion transport, trapping and detection (since the stability of the complex for each ligand in the gas phase may not follow that in solution, as already discussed). Secondly, differences in sensitivity due to ionization, ion transport and detection of the ligands should also be small. The approach assumes similar ionization efficiencies for complexes of different ligands, an assumption justified since these complex ions have very similar formation efficiencies due to the fact that the protein accounts for the bulk of the complexes' mass and charge. Consistent with this assumption, the observed charge states of the BCAII–inhibitor complexes are similar to those of the free BCAII ions.

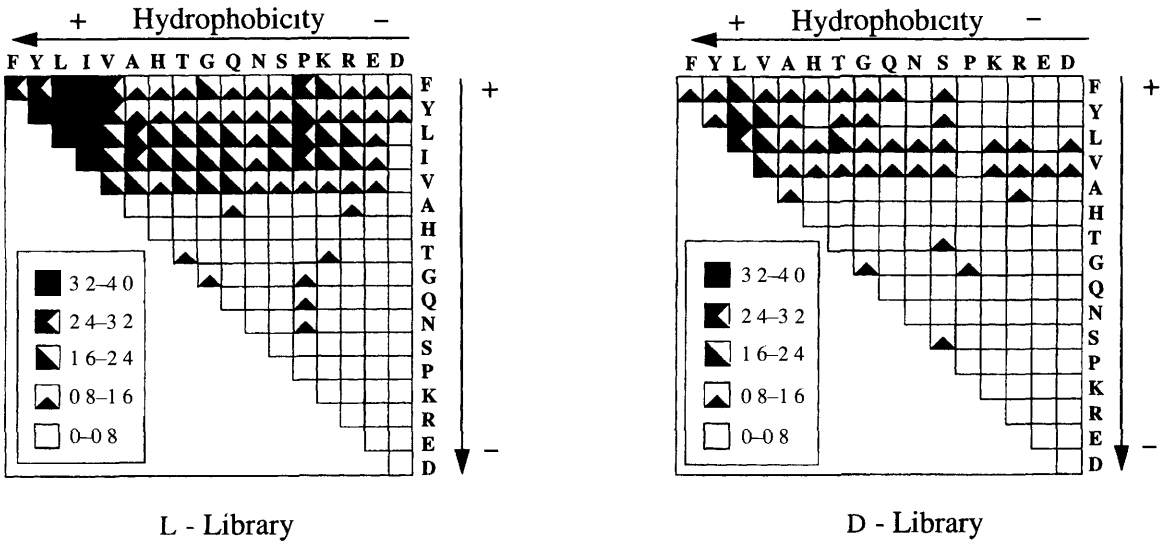


Fig. 10 The dependence of relative ion intensities on the composition of amino acids in the peptide library The structure of the peptide library is shown at the top of the figure The amino acids (AA_1 and AA_2) are arranged in such a way that their hydrophobicity decreases from left to right, and from top to bottom The relative ion intensity for each ligand was obtained by comparing the ion intensity of this ligand to that of the Gly Gly compound (present in both libraries) The results from L- and D-library were plotted on the same scale of relative ion intensity (bottom left corner) [33] The one letter codes for amino acids were used to indicate the identity of AA_1 and AA_2 (adapted from ref 33)

The ESI–FTICR approach we have described generates relative binding affinities for a large number of ligands simultaneously in one relatively fast experiment Fig 10 gives a representation of the relative abundances for the dissociated ligands in terms of the composition of the amino acids for the two libraries [Note that sequence isomers (–AA_x–AA_y– and –AA_y–AA_x–) and structural isomers (*e g* –Leu–Leu– and –Ile–Ile– in the L-library) are not distinguished since their molecular masses are identical] Seven individual inhibitors were then synthesized from the two libraries to determine their binding affinities to BCAII in solution using a fluorescence binding assay [33] The binding constants of the seven inhibitors in solution correlated well with the relative intensities of the ions dissociated from the BCAII–inhibitor complexes (Fig 11) The K_b for the tightest binding inhibitor identified by ESI–MS (AA_1 = AA_2 = L–Leu, see Fig 10) was $1\,4 \times 10^8$ dm^3 mol^{-1} The K_b of the compound bound most weakly (AA_1 = AA_2 = Gly) was $4\,9 \times 10^6$ dm^3 mol^{-1} The data also indicated that the addition of hydrophobic groups at the *para* position of benzenesulfonamide increases binding constants The chirality of the amino acids also appeared to influence the binding affinities of the tripetide inhibitors—the side chains of L-amino acids interact more effectively with the active site of CAII than did D-amino acids

The BACMS approach should be most effective for identification of the tight binding ligands, since lower abundances and contributions from nonspecific (random) associations may limit the quality of information for weaker binding ligands, particularly in larger libraries Further study of additional well-characterized model systems will be necessary to define the extent to which quantitative information can be derived from this method In principle, the ESI–FTICR methods should be most useful for determining the relative binding affinities for a library of compounds to a protein when (*a*) the binding studies can be carried out using 'volatile' buffers (*e g* ammonium acetate, Tris acetate, and ammonium hydrogencarbonate), (*b*) all the library components will be charged after dissociation of complexes in the gas phase, and (*c*) the library components have different masses The design of libraries in a way that ensures that each component has a different molecular mass can eliminate ambiguities in the identification of ligands and reduce the demands for subsequent dissociation and MS stages The fact that relative binding constants can be rapidly derived from these experiments for all or most of the members of a library may provide the basis for facile screening of combinatorial libraries, as well as a basis for the extension of this methodology for qualitative studies using more complex or poorly characterized libraries and even complex fractions derived from natural products The attractions of this approach include speed and the immediate characterization of the most relevant components from complex and otherwise poorly characterized mixtures Less obvious advantages include the potential for establishing difference in binding properties for biopolymer variants and modified biopolymers, in such cases the biopolymers would also be characterized by MS methods after dissociation of complexes

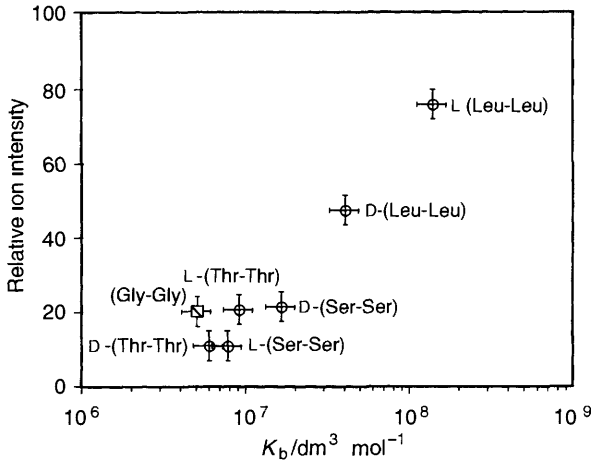


Fig. 11 Correlation of relative ion intensities *vs* K_b in solution for seven peptide inhibitors from the L- and D-library [33] We selected inhibitors having identical AA_1 and AA_2, since their ion intensities are free of ambiguities that are caused by sequence isomers (adapted from ref 33)

8 Concluding remarks

The application of ESI–MS for the study of noncovalent associations in solution constitutes a new capability with potentially broad applications in biochemical and biomedical research Establishing relative binding affinities using competitive binding experiments from measurements of relative abundances of the complexes in the gas phase is clearly feasible The detection of noncovalent complexes also provides a basis

for more conventional approaches, such as those involving the variation of ligand concentration and the use of Scatchard plots for determining binding constants.[34] These methods increasingly benefit from the rapid maturation of capabilities for accurate mass measurements and multi-stage MS methods for obtaining structural information. The full integration of these capabilities and continuing instrumental advances are certain to greatly expand the range of biopolymers and their complexes subject to study. For example, it should be feasible to study individual virus particles or large protein complexes by ESI–FTICR, and multi-stage MS studies of even individual molecules recovered from the surface of such complexes will also likely prove to be feasible. To the extent that the three dimensional solution structure is retained in the gas phase, a question clearly demanding additional study, H/D exchange and affinity (*i.e.* small molecule adduction) experiments conducted in the gas phase offer the potential for probing structure, and a basis for biophysical studies that can contribute to understanding protein folding. Such previously fanciful experiments are increasingly feasible based upon continuing refinement of electrospray methods and FTICR capabilities for sophisticated ion manipulations, indefinite trapping periods, high sensitivity, and the ability to make series of precise measurements from only a single ion.[3,35]

9 Acknowledgments

The authors wish to thank Drs H. R. Udseth, C. Liu, D. C. Muddiman, L. Pasa Tolic and S. A. Hofstadler, the early contributions of Dr X. Cheng (Abbott Laboratories) to this work and gratefully acknowledge the fruitful collaboration with Professor George Whitesides and co-workers at Harvard University. We thank the National Institutes of Health (GM53558) for support of this research. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the US Department of Energy, through Contract No. DE-AC06-76RLO 1830.

10 References

1 R. D. Smith, J. A. Loo, C. G. Edmonds, C. J. Barinaga and H. R. Udseth, *Anal. Chem.*, 1990, **62**, 882.
2 G. Siuzdak, B. Bothner, M. Yeager, C. Brugkdou, C. M. Faquet, K. Hoey and C. M. Chang, *Chem. Biol.*, 1996, **3**, 45.
3 R. D. Chen, X. H. Cheng, D. W. Mitchell, S. A. Hofstadler, Q. Wu, A. L. Rockwood, M. G. Sherman and R. D. Smith, *Anal. Chem.*, 1995, **67**, 1159.
4 R. D. Smith and K. J. Light-Wahl, *Biol. Mass Spectrom.*, 1993, **22**, 493.
5 R. D. Smith, X. Cheng, J. E. Bruce, S. A. Hofstadler and G. A. Anderson, *Nature*, 1994, **369**, 137.
6 A. Miranker, C. V. Robinson, S. E. Radford, R. T. Aplin and C. M. Dobson, *Science*, 1993, **262**, 896.
7 J. V. Iribarne and B. A. Thomson, *J. Chem. Phys.*, 1976, **64**, 2287.
8 M. Dole, L. L. Mack, R. L. Hines, R. C. Mobley, L. D. Ferguson and M. B. Alice, *J. Chem. Phys.*, 1968, **49**, 2240.
9 B. E. Winger, K. J. Light-Wahl, R. R. O. Loo, H. R. Udseth and R. D. Smith, *J. Am. Soc. Mass Spectrom.*, 1993, **4**, 536.
10 K. J. Light-Wahl, B. L. Schwartz and R. D. Smith, *J. Am. Chem. Soc.*, 1994, **116**, 5271.
11 E. R. Williams, *J. Mass Spectrom.*, 1996, **31**, 831.
12 M. S. Wilm and M. Mann, *Int. J. Mass Spectrom. Ion Proc.*, 1994, **136**, 167.
13 X. Cheng, A. C. Harms, P. N. Goudreau, T. C. Terwilliger and R. D. Smith, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 7022.
14 S. L. Kaufman, J. W. Skogen, F. D. Dorman, F. Zarrin and K. C. Lewis, *Anal. Chem.*, 1996, **68**, 1895.
15 R. D. Smith, J. E. Bruce, Q. Wu, X. Cheng, S. A. Hofstadler, G. A. Anderson, R. Chen, R. Bakhtiar, S. O. Van Orden, D. C. Gale, M. G. Sherman, A. L. Rockwood and H. R. Udseth, *Mass Spectrometry in the Biological Sciences*, 1996, ed. A. Burlingame and S. Carr, Humana Press, Totowa, NJ, 25.
16 M. B. Comisarow and A. G. Marshall, *Chem. Phys. Lett.*, 1974, **25**, 282.
17 J. P. Speir, G. S. Gorman, C. C. Pitsenberger, C. A. Turner, P. P. Wang and I. J. Amster, *Anal. Chem.*, 1993, **65**, 1746.
18 S. A. Hofstadler, F. D. Swanek, D. C. Gale, A. G. Ewing and R. D. Smith, *Anal. Chem.*, 1995, **67**, 1477.
19 J. C. Severs, S. A. Hofstadler, Z. Zhao, R. T. Sehn and R. D. Smith, *Electrophoresis*, 1996, **17**, 1808.
20 K. J. Light-Wahl, R. D. Smith and B. E. Winger, *J. Am. Chem. Soc.*, 1993, **115**, 5869.
21 R. D. Smith, K. J. Light-Wahl, B. E. Winger and J. A. Loo, *Org. Mass Spectrom.*, 1992, **27**, 811.
22 B. L. Schwartz, K. J. Light-Wahl and R. D. Smith, *J. Am. Soc. Mass Spectrom.*, 1994, **5**, 201.
23 B. L. Schwartz, D. C. Gale, R. D. Smith, A. Chilkoti and P. S. Stayton, *J. Mass Spectrom.*, 1995, **30**, 1095.
24 D. C. Muddiman, D. S. Wunschel, C. Liu, L. Pasa-Tolic, K. F. Fox, A. Fox, G. A. Anderson and R. D. Smith, *Anal. Chem.*, 1996, **68**, 3705.
25 M. J. Greig, H. Gaus, L. L. Cummins, H. Sasmor and R. H. Griffey, *J. Am. Chem. Soc.*, 1995, **117**, 10765.
26 X. Cheng, P. E. Morin, A. C. Harms, J. E. Bruce, Y. Ben-David and R. D. Smith, *Anal. Biochem.*, 1996, **239**, 35.
27 X. Cheng, R. Chen, J. E. Bruce, B. L. Schwartz, G. A. Anderson, S. A. Hofstadler, D. C. Gale, R. D. Smith, J. Gao, G. B. Sigal, M. Mammen and G. M. Whitesides, *J. Am. Chem. Soc.*, 1995, **117**, 8859.
28 J. Gao, G. M. Whitesides, Q. Wu, P. Lei and R. D. Smith, unpublished work, 1996.
29 Q. Wu, J. Gao, G. B. Sigal, J. E. Bruce, G. M. Whitesides and R. D. Smith, *J. Am. Chem. Soc.*, 1997, **119**, 1157.
30 J. E. Bruce, G. A. Anderson, R. D. Chen, X. H. Cheng, D. C. Gale, S. A. Hofstadler, B. L. Schwartz and R. D. Smith, *Rapid Commun. Mass Spectrom.*, 1995, **9**, 644.
31 J. E. Bruce, S. L. Van Orden, G. A. Anderson, S. A. Hofstadler, M. G. Sherman, A. L. Rockwood and R. D. Smith, *J. Mass Spectrom.*, 1995, **30**, 124.
32 J. E. Bruce, G. A. Anderson and R. D. Smith, *Anal. Chem.*, 1996, **68**, 534.
33 J. Gao, X. Cheng, R. Chen, G. B. Sigal, J. E. Bruce, B. L. Schwartz, S. A. Hofstadler, G. A. Anderson, R. D. Smith and G. M. Whitesides, *J. Med. Chem.*, 1996, **39**, 1949.
34 H. K. Lim, Y. L. Hsieh, B. Ganem and J. Henion, *J. Mass Spectrom.*, 1995, **30**, 708.
35 J. E. Bruce, X. Cheng, R. Bakhtiar, Q. Wu, S. A. Hofstadler, G. A. Anderson and R. D. Smith, *J. Am. Chem. Soc.*, 1994, **116**, 7839.

Received, 23rd December 1996,
Accepted, 14th March 1997