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PERSPECTIVES

^{252}Cf-Plasma Desorption Mass Spectrometry II—A Perspective of New Directions

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INTRODUCTION

With close to 200 ^{252}Cf-plasma desorption mass spectrometry (^{252}Cf-PDMS) systems now in operation, novel new applications, insights into mechanisms and improvements in performance are appearing as more scientists become involved with the technique. The objective of this Perspectives II article is briefly to report on some of these new developments and to describe in more detail projects that portend future directions for ^{252}Cf-PDMS.

APPLICATIONS

Fragmentation analysis using the primary mass spectrum

Fragmentation pattern analysis to obtain sequence information by ^{252}Cf-PDMS was first demonstrated by Chait and Field almost a decade ago.[1] Interest in this activity was renewed by work of the van der Greef group in Holland in 1990[2] which showed that the *primary* ^{252}Cf-PD mass spectrum contains sequence information for peptides up to 4.6 kDa. Bunk *et al.*, following these findings, were able to extend these measurements for proteins up to 30 kDa.[3] The significance of this renewed activity is that it is now possible to obtain detailed fragmentation data using a simple single-stage ^{252}Cf-PDMS system. An overall picture of the fragmentation process has emerged from the systematic studies that Bunk has carried out. First, arginine residues are the dominant protonation site and cleavage occurs via the Gross charge-remote fragmentation mechanism.[4] The fragmentation pathways that are excited include those associated with high molecular excitation (side-chain cleavages and immonium ion formation requiring the cleavage of two peptide bonds). The influence of the presence of disulfide linkages on the fragmentation of a series of cystine-containing peptides (vasopressin, somatostatin, atrial natriuretic peptide and insulin) has shown that fragmentation suppression occurs within cystine loops and the partitioning of fragmentation within the molecule is influenced by the presence of these loops.[5] Insulin is one of the molecules that was studied. Figure 1(a) shows a ^{252}Cf-PD mass spectrum in the region m/z 1400–3000 where N- and C-terminal fragment ions are observed coming from cleavage occurring within the cystine loop of the B-chain. Note that a fragment radical cation $A^{+\cdot}$ is observed from the A chain which is a reflection of the absence of a strong protonation site in that part of the molecule. Figure 1(b) shows how the pattern changes when the sample is reduced *in situ* using dithiothreitol (DTT). A much more extensive fragmentation pattern is observed from the B-chain. Figure 1(c) shows the fragmentation pattern for the intact insulin in the high-mass region. Fragment ions resulting from cleavage around the Arg residue near the C-terminal domain of the B-chain are observed with the A-chain still attached to the fragment ion.

The development of ^{252}Cf-PDMS for fragmentation pattern analysis in the studies carried out at Texas A&M was facilitated by improvements in the performance of the ^{252}Cf-PD instrument as well as in data analysis. The flight tube was lengthened to 1 m and a 78 ps time interval was used in acquiring the time-of-flight (TOF) data. By a careful selection of choice of calibration ions and utilizing a computer-interactive algorithm for centroid determination, m/z values with mass accuracies at the 200 p.p.m. level were achieved. Table 1 shows the mass data obtained for the fragmentation of oxidized and reduced atrial natriuretic peptide that demonstrates the level of accuracy that has been achieved.

The group headed by G. Allmaier at the University of Vienna, using a Bio-Ion ^{252}Cf-PDMS system, has found that the broad range of internal excitations characteristic of ^{252}Cf-PDMS leads to the generation of extensive sequence-specific fragmentation patterns for peptidoglycan monomers: species isolated from peptidoglycan heteropolymer units from bacterial cell walls. An example of the structure of one of these monomer units is shown in Fig. 2 along with the fragmentation pattern. Most fragment ions of the carbohydrate as well

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as the peptide subunit correspond to charge-remote fragmentations due to the 'C-terminal' Lys-Arg-dipeptide moiety. The peptide subunit of this peptidoglycan monomer shows peptide linkage inversion between diaminopimelic acid and lysine of the type $A_2pm \rightarrow \varepsilon$-Lys. A novel feature of the ^{252}Cf-PD mass spectrum of this molecule is that the charge-remote fragmentation extends into the carbohydrate domain of this molecule, a pathway that may become accessible under the conditions of high energy density and short excitation time that is a unique feature of the ^{252}Cf-PD process.

^{252}Cf-PDMS used in the study of antigen–antibody interactions

No subject is more rich in current medical research than the study and control of the human immune system. Basic to its function is the primary interaction,

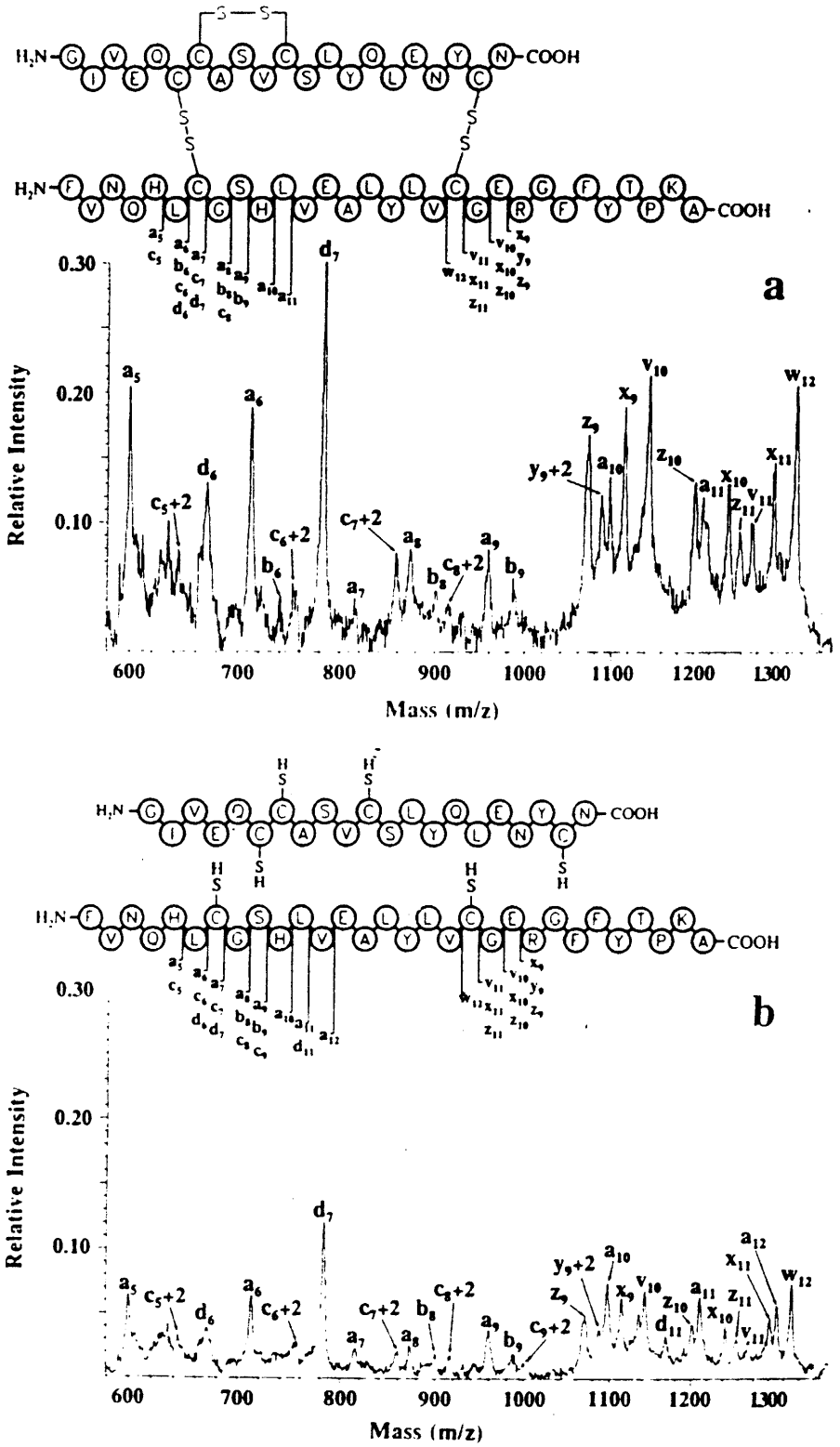

Figure 1. Continued on facing page.

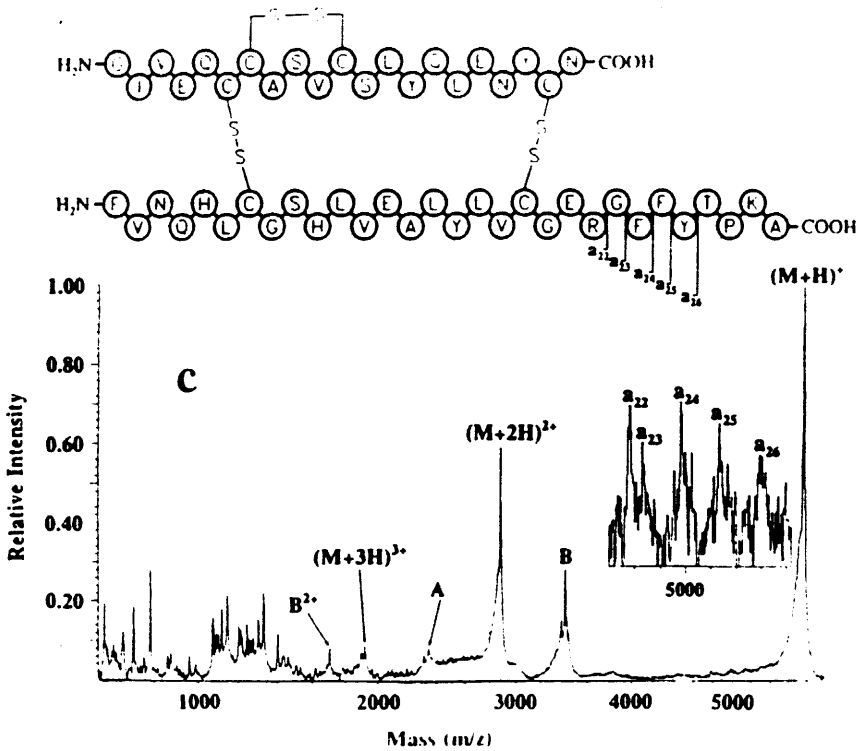

Figure 1. ^{252}Cf-PD mass spectrum of insulin: (a) fragmentation pattern in the intermediate mass region for the oxidized form and (b) the reduced form showing the influence of the reduction of the disulfide bridges on the fragmentation pattern of the B-chain domain; (c) wider mass range for the oxidized insulin spectrum showing fragment ions containing the intact A-chain. Reprinted with the permission of Elsevier.

the identification of a molecule as an antigen and the expression of an antibody to bind and biologically neutralize the antigen. Molecular details of this interaction are now being unraveled, including the identification of the specific sites on an antigen that are recognized by an antibody as binding domains: the epitope regions of the antigen. Recent studies on the action of proteolytic enzymes on antibodies and immune complexes open up the possibility that mass spectrometry may play an important role in learning some of the details of the

Table 1. Comparison of observed and calculated masses of molecular and prominent fragment ions of oxidized and reduced atrial natriuretic peptide

Ion	Calculated mass[a] (u)	Oxidized Observed mass (u)	Reduced Observed mass (u)	Ion	Calculated mass[a] (u)	Oxidized Observed mass (u)	Reduced Observed mass (u)
$[M + H]^+$	3063.5	3063.7	3065.7	d_{13}	1364.6	1364.1	1364.7
	3065.5			a_{13}	1408.6	1408.0	1408.8
$[M + 2H]^{2+}$	1532.2	1532.5		a_{14}	1564.8	1564.4	1564.7
	1533.2		1533.3	a_{15}	1678.0	1677.2	1677.7
a_3	329.2	329.3	329.3	a_{16}	1735.0	1734.3	1734.7
d_4	400.5	400.5	400.5	a_{17}	1806.1	1805.3	1805.8
a_4	485.6	485.5	485.4	a_{18}	1934.2	1933.3	1934.0
d_5	556.7	556.5	556.4	a_{19}	2021.3	2021.0	2020.9
a_5	572.7	572.5	572.5	a_{21}	2191.5	2190.4	2191.4
d_6	643.8	643.7	643.6	a_{22}	2248.6	2247.4	2247.8
a_6	659.8	659.7	659.8	a_{24}	2463.8	2463.8	
d_7	730.8	730.8	730.7		2465.8		2466.0
a_7	762.9	762.8	762.9	d_{25}	2534.9	2534.4	
a_8	910.1	910.2	910.1		2536.9		2536.1
a_9	967.1	967.2	967.5	a_{25}	2550.9	2550.6	
a_{10}	1024.2	1023.7	1024.4		2552.9		2552.4
d_{11}	1095.3	1095.3	1095.7	a_{26}	2698.1	2698.3	
a_{11}	1180.4	1180.0	1180.6		2700.1		2700.5
a_{12}	1293.5	1293.1	1293.6	a_{27}	2854.3	2853.8	
					2856.3		2856.6

[a] Isotopically averaged mass

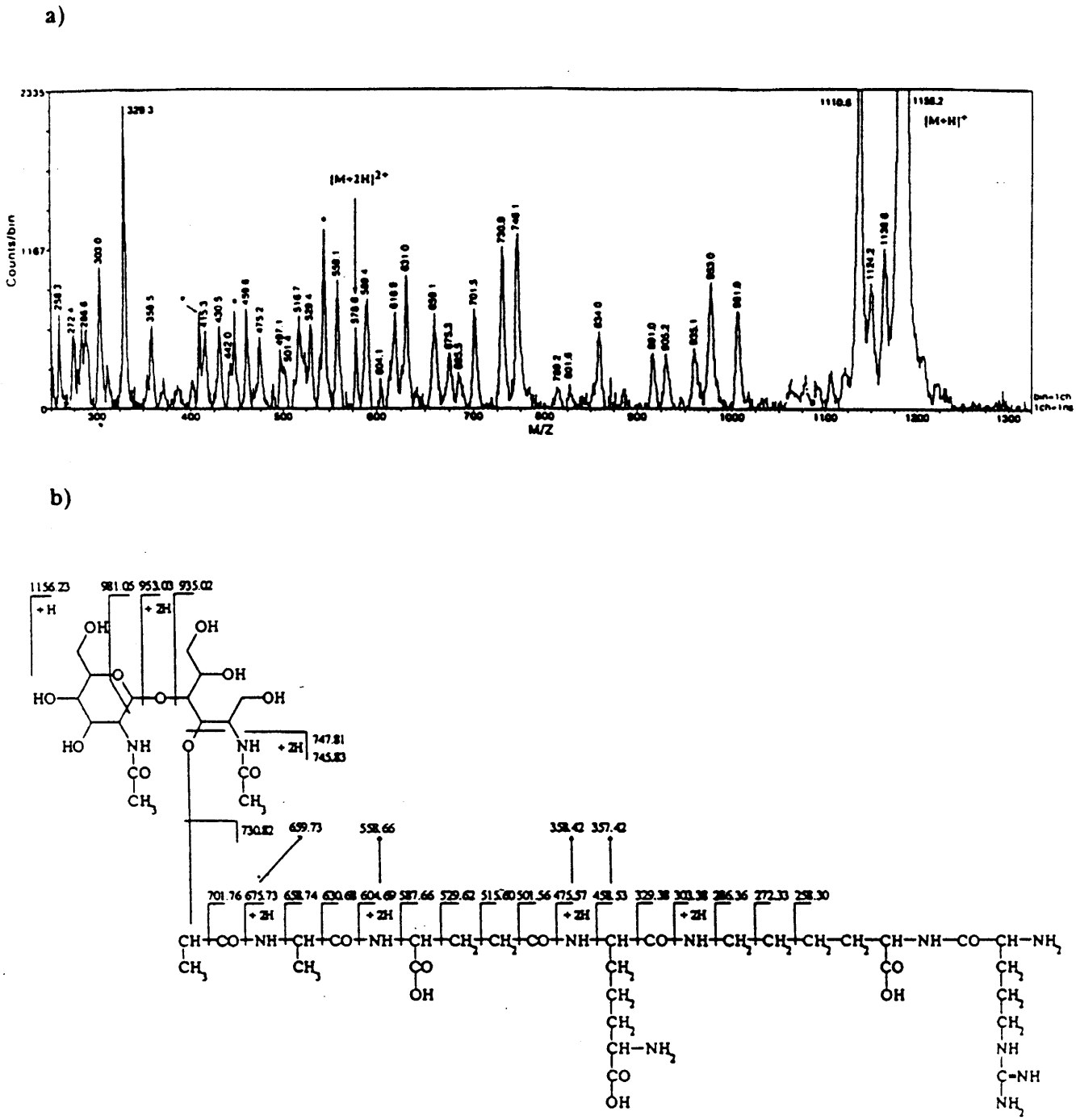

Figure 2. ^{252}Cf-PD mass spectrum of a peptidoglycan monomer. (a) the positive ion spectrum showing an extensive fragmentation pattern. (b) the molecular structure with most of the fragment ions identified.

immune response at the molecular level. The feasibility of this idea has now been proven in the laboratory of M. Przybylski at the University of Konstanz and ^{252}Cf-PDMS was used as the primary mass spectrometric method.[6]

Antibodies are resistant to proteolysis, a property that provides *in vivo* protection of these molecules. When an antibody binds to an antigen, the epitope regions of the immune complex become inaccessible to proteolytic cleavage. Suckau *et al.* used these features to identify the molecular epitope domains of complement component C3a, a potent protein mediator of the inflammatory response.[6] The scheme that was used is outlined in Fig. 3 and involves three separate experiments. First, the antigen is subjected to proteolytic digestion and the fragments are analyzed by mass spectrometry to give a 'peptide map' of all the domains that are excised from the antigen by the enzyme. In the second experiment, the digestion is repeated for the immune complex. Only the non-epitope regions of the antigen are exposed to the enzyme so that the excised domains that appear in the second map are from these non-epitope regions of the antigen. The third experiment provides a check on the validity of the epitope domain identification. After proteolytic digestion of the immune complex, the residual complex comprises the

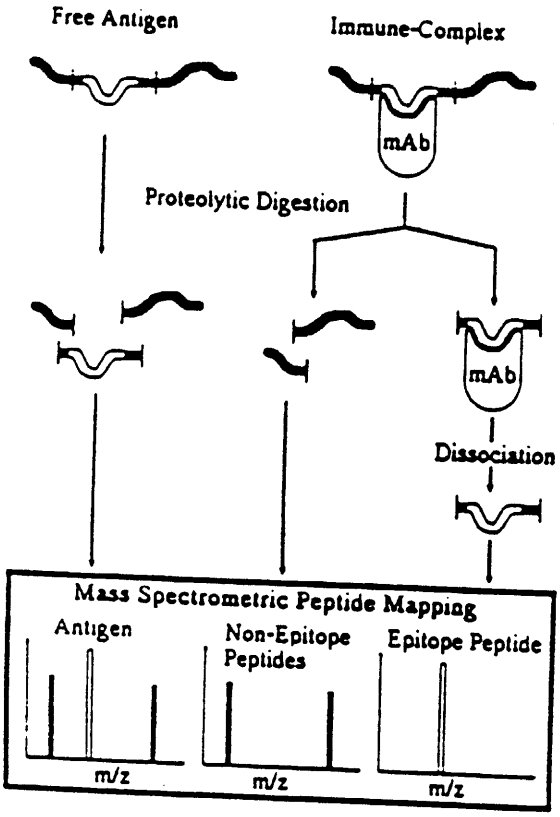

Figure 3. Schematic of the mass spectrometric epitope mapping method of an antigen–antibody complex as compared to peptide mapping of the free antigen. Molecular ions of non-epitope and epitope proteolytic peptides are illustrated by solid and open bars respectively. Reprinted with the permission of the US National Academy of Sciences.

antibody and the epitope regions of the antigen bound to the antibody. This complex is then dissociated by increasing the ionic strength of the solution and a mass spectrometric peptide map is recorded for the epitope fragments.

What made ^{252}Cf-PDMS attractive for this study was the fact that serial measurements can readily be carried out. After proteolysis, the peptide digest was purified by high-performance liquid chromatography (HPLC). A series of nitrocellulose targets was prepared prior to the separation. As each fraction passed through the HPLC detector, a single droplet was collected on one of the nitrocellulose targets. At the end of the experiment, these targets, up to 12, were then inserted into a Bio-Ion ^{252}Cf-PDMS system and the targets were analyzed sequentially. An important feature of this approach is that the sample fraction is not destroyed in the analysis. Thus, any target can be reanalyzed for a longer time period or using a different polarity acceleration voltage to record a negative ion spectrum. In addition, the samples prepared in this manner have a low level of contamination, so that there is little quenching from matrix effects.

Quantitating ^{252}Cf-PDMS

Molecular mass determination and fragmentation patterns are the mainstream activity of mass spectrometry. Determination of the concentration of an analyte in a sample by mass spectrometry is considerably more difficult, particularly when the analyte is involatile. Since a wide variety of pharmaceuticals fall into this category, the development of quantitative mass spectrometry for these species is of considerable importance. The main problem in quantitation using solid matrices is that molecular ion formation is sensitively dependent on the composition of the matrix and the presence of impurities.

The group of H. Jungclas at the Marburg Medical School was the first to develop ^{252}Cf-PDMS for monitoring of therapeutic drug levels in human plasma.[7] Noteworthy in their approach to the solution of the problem was that they were guided by fundamentals of the desorption/ionization process that were previously established in other studies. Specifically, a significant component of the species desorbed in ^{252}Cf-PDMS consists of large metastable clusters of molecules held together by van der Waals forces that rapidly dissociate close to the surface of the sample. Molecular ions of the analyte are formed from the dissociation of these metastable molecular clusters.

Jungclas postulated that the chemistry that takes place within these clusters determines the nature and intensity of the molecular ions that appear in the ^{252}Cf-PD mass spectrum. If the cluster contains impurities, they could react with the analyte molecules within the cluster either to enhance or quench molecular ion formation. Thus, with no control of the composition of the cluster, it is not possible to develop a quantitative assay. Jungclas solved this problem by controlling the chemical reactions in the metastable clusters using a matrix isolation approach where the analyte molecules are a small fraction of the total composition of the cluster. The matrix molecules are to serve several purposes: to isolate the analyte molecules from each other but forming a shell of weakly interacting volatile molecules around the analyte molecule. When required, scavenger molecules are added that bind strongly to impurities in the matrix. Thus, the isolating matrix is a 'designer matrix' whose physical/chemical properties are matched with the chemistry of the analyte and with a scavenger component tailored to the chemistry of known interfering impurities in the sample.

The procedure developed is quite straightforward. First, the serum or urine sample is spiked with a mass spectrometric standard, a molecule which is very close chemically to the analyte. Then, the fraction containing the analyte and standard is isolated as a crude extract using thin-layer chromatography (TLC) or chromatographic cartridges (e.g. SEP-PAK C-18). The isolating matrix molecules are then added to the solution in 10–1000 molar excess and an aliquot of the solution is evaporated to dryness on a thin sample backing and analyzed by ^{252}Cf-PDMS.

The first quantitative ^{252}Cf-PD mass analysis that was developed was for serum levels of etoposide, a cytostatic drug used in the treatment of germ cell tumors and lymphoma.[7] The analog molecule used for quantitation was teniposide, whose structure is similar to the analyte, varying in one side-chain substituent. A ^{252}Cf-PD mass spectrum of a crude extract from blood plasma spiked with close to equal molar amounts of

analyte and standard is shown in Fig. 4(a). Weak peaks from these molecules were detected as $M^{+\cdot}$ ions along with $[M + Na]^+$ adduct ions. Examining samples with different molar ratios of analyte standard showed that quantitation was not being achieved.

At this point, a 'designer matrix' was developed to match the solubility properties of the analyte standard, the chemistry of the ion formation process, radical cation formation, and the presence of alkali metal salts in the sample. A mixture of methenamine and sucrose octaacetate was used. The sucrose octaacetate was chosen for its ability to scavenge alkali metal ions and methenamine to surround the analyte molecule with a stable, volatile, chemically inert protective sheath. Figure 4(b) shows the influence of this matrix on the etoposide/teniposide spectrum. Even though the mole percentage of analyte has been reduced to 3%, the molecular ion intensity has more than doubled, the alkali metal ion adducts have disappeared, and the background peaks have been greatly suppressed. Increasing the mole percentage of methenamine by a factor of three suppresses the background peaks even further and, remarkably, with no reduction in the analyte/standard ion intensities (Fig. 4c). Using this matrix, a quantitative analysis for etoposide was developed and is being used in clinical applications. The calibration curve (analyte/standard intensity ratios versus concentration) is linear for over three orders of magnitude etoposide concentration in blood plasma.

Choice of designer matrix is very much dependent on the properties of the analyte, the gas-phase reactions that produce the molecular ion and the influence of impurities. Recently, a ^{252}Cf-PDMS-based assay for plasma levels of doxorubicin was developed by the same group where the designer matrix was benzidine.[8]

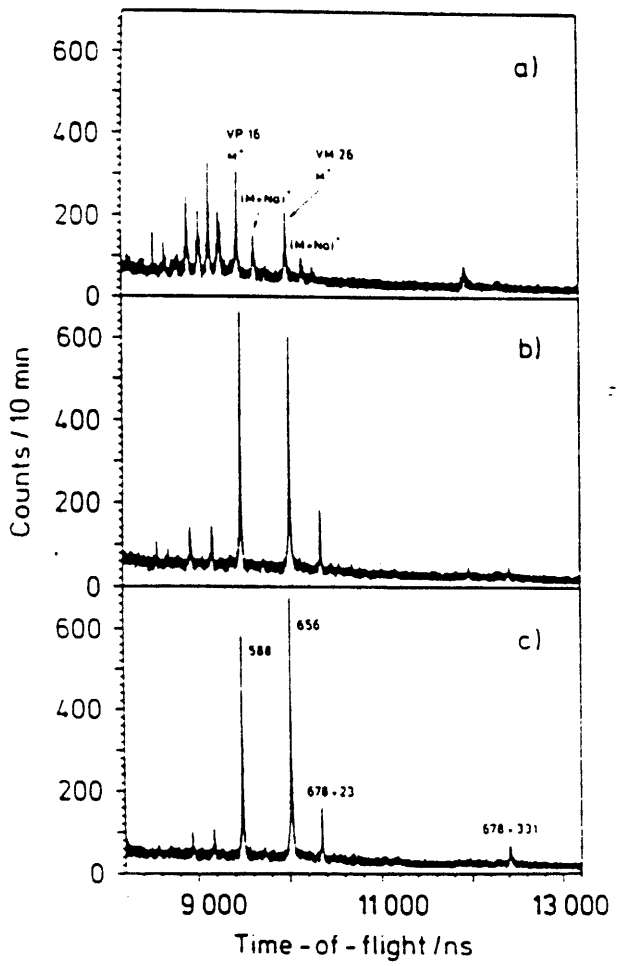

Figure 4. ^{252}Cf-PD mass spectra of etoposide (VP16) analyte and teniposide (VP26) standard used in quantitating serum levels of etoposide: (a) serum sample, separated by TLC, (b) after addition of 100 μg sucrose octaacetate and 50 μg methenamine as an isolated matrix, (c) after further treatment with an additional 100 μg methenamine. Reprinted with the permission of the authors.

Chemical microhomogeneity analysis using ^{252}Cf-PDMS

Microscopic analysis of thin tissue slices gives revealing detail of the structural architecture of cells: a collage of vesicles and membrane boundaries. Often these vesicles are pools of a biologically active agent that is secreted on-signal by the cell. To be able to measure the composition of a vesicle within a cell, to determine which components of a cell are coupled to each other, and which are not, i.e. chemical homogeneity within intracellular domains of a cell, would be a considerable achievement for bioanalytical chemistry. If, for example, a bacterial cell were being studied for chemical microhomogeneity using this technique, K^+ and Na^+ ions in the cytosol would appear as strongly correlated ions in the ^{252}Cf-PD mass spectrum; if an Na^+ ion is desorbed by a fission fragment, a K^+ ion is also desorbed at the same time because both ions are in close proximity in the cytosol. Molecular ions of phospholipids from the cell wall would be correlated with each other because they are in close proximity on a nanometer scale in the cell wall but would be only weakly correlated with Na^+ and K^+ ions. Mass spectrometry, and specifically, ^{252}Cf-PDMS, is showing promise as a method for chemical microhomogeneity analysis with sampling dimensions of 5–10 nm based on recent achievements of the group of E. Schweikert at Texas A&M University.[9,10]

The development of this application, as in the previously described application, was also predicated on a knowledge of the fundamental processes associated with ^{252}Cf-PDMS and the fact that the measurement is made at the single-event level: a single incident primary ion and a few ejected secondary ions.

When a fission fragment passes through a thin film, approximately 1000 molecules are ablated from the surface,[11] leaving a 5–10 nm diameter crater. Molecules of the matrix that reside within these dimensions will be ejected together. Those that reside outside the crater will be ejected in another primary excitation event along with neighbouring molecules that are within the excitation zone. Two or more ions that are formed by the same fission fragment excitation event are said to be *correlated* due to the fact that they were close to each other in the matrix. The correlation can be readily measured using a *coincidence counting technique* first described by the group of LeBeyec at Orsay.[12]

Chemical microhomogeneity studies using ^{252}Cf-PDMS have yet to be carried out using single cell matrices. But the principle and feasibility of the method have recently been demonstrated in a ^{252}Cf-PDMS

study using polystyrene/poly(vinyl methyl ether) (PS/PVME) as matrix model.[10] Two sample types were studied: one that was a homogeneous mixture of the two polymers and the other a phase-separated blend with chemical microheterogeneity at the 100 nm level. The ^{252}Cf-PD mass spectra of the individual components are shown in Fig. 5. The fingerprint of PS is a set of fragment ions of which ions at m/z 91 and 193 are the most distinctive. For PVME, ions at m/z 75, 85 and 101 are the signature of that domain. Figure 6(a) shows the ^{252}Cf-PD mass spectrum for a physical mixture of particulates of both polymers. The characteristic fragment ions from both polymers are present in the spectrum. Figure 6(b) shows the 'coincidence spectrum' recorded at the same time as the spectrum shown in Fig. 6(a). The only difference is that the spectrum is a subset of the spectrum shown in Fig. 6(a): an accumulation of ions where an m/z 101 ion was a component of the plume of species ejected from the fission track. If it was not, the ions from that plume did not contribute to that spectrum. The prominent ions are identical to what was observed for pure PVME. Thus, this spectrum is from the PVME particulates in the mixture that were struck by fission fragments. At the time that the software-controlled parameters were set to establish the coincidence windows, a window was also set at m/z 91, a fragment characteristic of PS. Figure 6(c) shows the m/z 91 coincidence spectrum, also a subset of the complete spectrum shown in Fig. 6(a), which features the

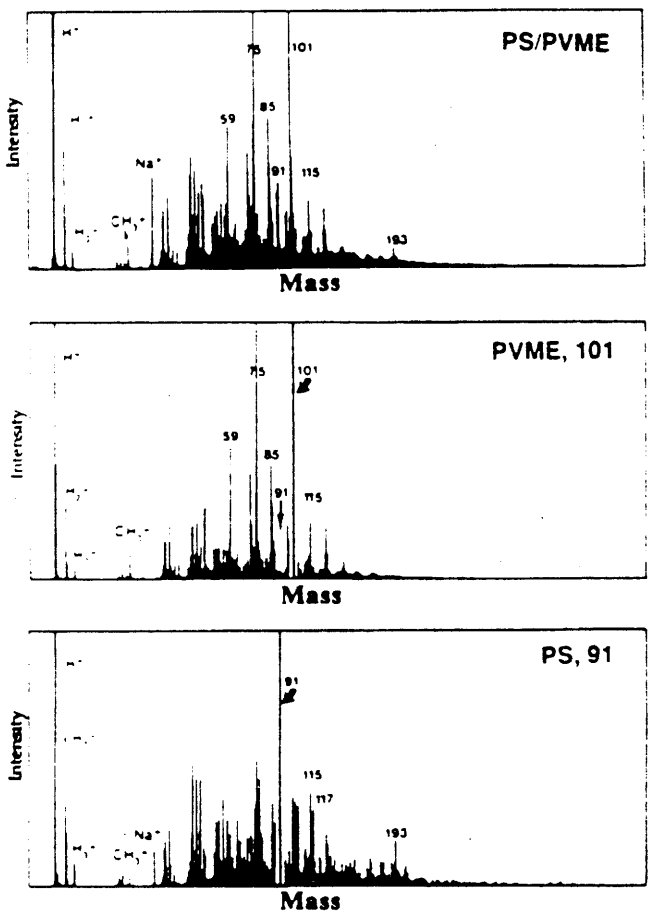

Figure 5. ^{252}Cf-PD mass spectra of two pure polymer films: (a) poly(vinyl methyl ether) and (b) polystyrene. Reprinted with the permission of the authors.

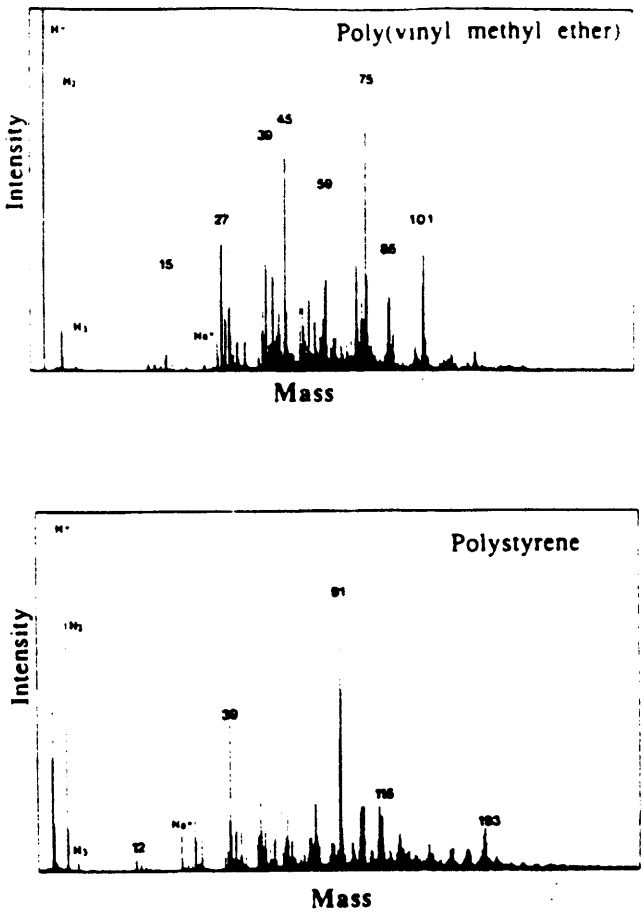

Figure 6. ^{252}Cf-PD mass spectra of a mixture of poly(vinyl methyl ether), (PVME) and polystyrene (PS), where the two domains are physically separated: (a) the total spectrum of the mixture; (b) spectrum of ions ejected in coincidence with the m/z 101 fragment ion of PVME; (c) spectrum of ions ejected in coincidence with the m/z 91 fragment ion of PS. Reprinted with the permission of the authors.

fragment ions characteristic of the PS particulates in the physical mixture. The observation that no PVME fragment ions are observed in the PS subset spectrum and no PS fragment ions in the PVME subset spectrum is the signature that the two phases are physically separated, the correct answer for this matrix. What happens when the two components form a solid homogeneous solution?

The degree of correlation has been quantitated in terms of ratios of intensities giving a correlation coefficient, Q, which is defined in reference 10. When $Q > 1$, for a particular binary ion set (A, B), the ions are correlated; if $Q < 1$, the ions are anti-correlated (the two ions originate from distinctly different domains); when $Q = 1$, A and B are randomly distributed throughout the matrix. Figure 7 is a correlation diagram for the PS/PVME system using the m/z 101 ion from PVME as the coincidence window. For the phase-separated matrix, correlation coefficients of 1.4–1.6 are obtained for those fragment ions that are part of the PVME spectrum, while the ions associated with PS are anti-correlated ($Q < 1$). For the miscible blend, the Q values approach unity for both families of ions, a signature that the sample is approaching a randomly distributed blend of the two components.

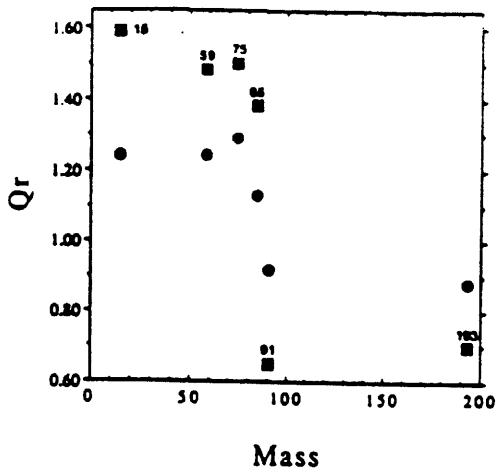

Figure 7. Relative correlation coefficient, Q_r, versus secondary ion mass using the *m/z* 101 ion intensity as the standard. The points labelled (●) are from the data for the miscible sample; the points labelled (■) are from the phase-separated sample. Reprinted with the permission of the authors.

The measurement of chemical microheterogeneity fully utilizes many of the unique aspects of ^{252}Cf-PDMS. This kind of analysis can be carried out on any ^{252}Cf-PDMS system. The only changes required to perform this analysis on a Bio-Ion ^{252}Cf-PD system would be in the software that controls the interface between the time-interval digitizer and the data acquisition system.

FUTURE DEVELOPMENTS AND APPLICATIONS OF ^{252}Cf-PDMS

Some general comments

The selected examples described in the previous section are representative of the link between past and future developments and applications of ^{252}Cf-PDMS. As the new techniques of matrix-assisted laser desorption ionization (MALDI)[13] and electrospray ionization (ESI)[14] continue to evolve in both understanding and application, the research objectives will also become more ambitious as we strive to improve the status of mass spectrometry as a significant analytical technique for the life sciences. The common feature for those few laboratories which utilize MALDI, ESI and ^{252}Cf-PDMS, e.g. the Roepstorff group at Odense, the van der Greef group at Leiden and the Przybylski group at Konstanz, is that the focus is on the structure and function of biological systems, and which is the 'best' mass spectrometric method is irrelevant. The three techniques are found to be complementary in these settings.

Future applications of mass spectrometry will involve more function studies where mass spectrometry will be used in kinetic studies, to study the evolution of a chemically reacting system, identify reaction intermediates, and give microscopic detail of locations of active sites in proteins. A recent Perspective in Biological Mass Spectrometry by Smith[15] on the use of ESI to study non-covalent complexes of proteins is indicative of the aspirations of those dedicating their efforts to contribute to the future and health of the field of mass spectrometry. *Perhaps the greatest challenge facing the biological mass spectrometry community is to develop mass spectrometric methods of analysis that faithfully measure the dynamics of* in vivo *biological processes.* Does what occurs in solution have any relevance to what is observed in the mass spectrometer when the analyte is extracted from an aqueous environment and transformed into a gas-phase ion in high vacuum? We face a challenge to overcome the general bias and skepticism of a wary community of life scientists whose first reaction to an unexpected observation from a mass spectrometric measurement is that it is an *artifact* of the technique. In the following sections, two topics have been selected as representative of future developments and applications of ^{252}Cf-PDMS. The first of these addresses the problem of correlating aqueous solution chemistry and ^{252}Cf-PD mass spectra.

How can we study aqueous solution chemistry using ^{252}Cf-PDMS?

The basic problem in using mass spectrometry to study the interactions of species in aqueous solution is that water, through hydrogen bonding, ion–dipole interactions and dielectric shielding, has a dominating influence on the equilibrium dynamics. To record a mass spectrum, gas-phase molecular ions must be formed, free of the solvation sphere surrounding these species in solution and from gas-phase reactions which could alter the molecular structure of the ions. So how do we sample a solution in dynamic chemical equilibrium and identify the components of the equilibrium by mass spectrometry without losing or distorting the chemical details? The solution to the problem is part of the future research and development of ^{252}Cf-PDMS. What is described below is a first attempt at solving the problem.

Capturing the components of a chemical equilibrium at a solid/solution interface. To illustrate the principles being proposed, let us use as a model—a well-characterized chemical system—the interaction of angiotensin II (ang II) with Cu(II) in aqueous solution.[16] From potentiometric titrimetry and nuclear magnetic resonance (NMR), two Cu(II) binding sites have been identified, one at the N-terminus with the N-terminal amine serving as the anchoring site. The other anchoring site is at the side-chain imidazole on His.[6] The stability of the complex is pH dependent because, at both sites, deprotonated amide nitrogen atoms in the peptide bond are part of the coordination sphere that stabilizes the complex. Thus, at low pH ang II exists as the free peptide in the presence of Cu(II), but at neutral pH a stable Cu complex is formed, predominantly involving the N-terminal region.

What is going on in solution at equilibrium? Cu(II) is kinetically active in the complex. It is rapidly exchanging with H^+ (aq.) and Cu^{+2} (aq.) at both sites so that in any instant a population of various forms of aqueous ang II molecular ions exists and this population is pH

dependent. We would like to sample this equilibrium mixture without significantly perturbing the equilibrium or the nature of the species involved. One approach being developed is to capture these species from solution onto a solid surface which has a high affinity for the peptide components of the equilibrium mixture. What happens when the solution is suddenly separated from the solid surface? Does the population of adsorbed ang II molecules resemble what is present in solution? This approach is being studied by Zhaohong Hu at Texas A&M as part of her PhD dissertation studies.[17]

The first test of the relevance of a ^{252}Cf-PD mass spectrum of ang II + Cu(II) to the aqueous solution dynamics was the study of the pH dependence. When ang II is mixed with Cu(II) and the solution is adjusted to a pH of 3, there is no [ang II + Cu(II)] complex in the solution and the composition is essentially protonated ang(II) and solvated Cu(II). As the solution pH is increased, the Cu(II)/peptide complex begins to form under these conditions and becomes a significant component of the population of ang II-related species. The mass spectrum of species adsorbed onto nitrocellulose should reflect this pH dependence. This feature is what was observed and is depicted in Fig. 8. The protonated ang II molecular ion is the most intense peak in the molecular ion region at low pH, while the Cu complex, $[M + Cu - H]^+$, becomes the dominant species at higher pH. In previous studies, we found that Arg^2 is the protonation site for this molecule[3] and the fragmentation pattern for the Cu complex confirms that this charge site is retained in the Cu complex. Thus, the coordination of Cu(II) to ang II is associated with the loss of two protons, which confirms the findings from solution studies.[16] This result demonstrates the ability of mass spectrometric analysis to obtain information on the contribution of deprotonated ligands in the formation of the coordination complex.

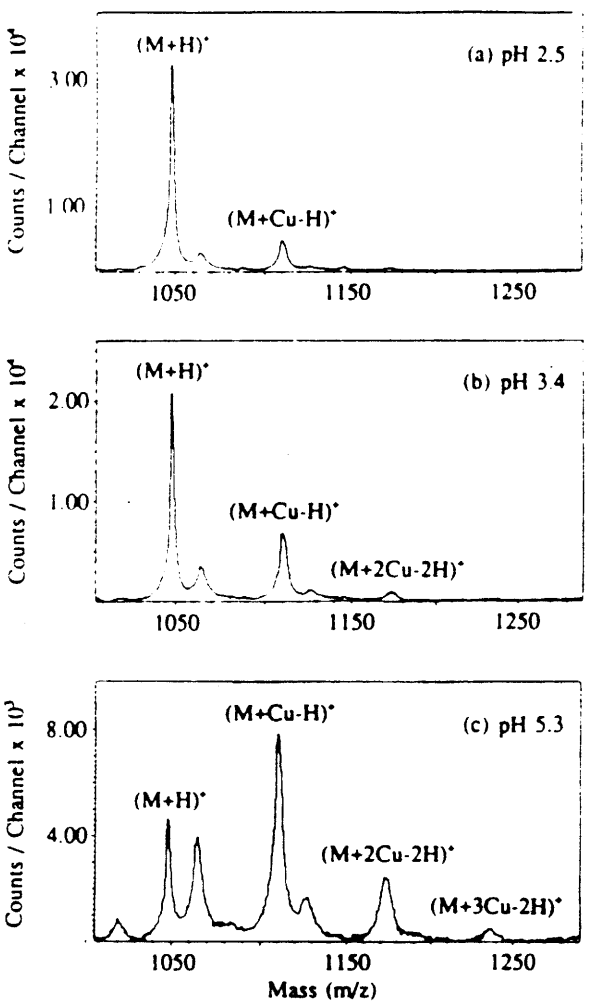

Figure 8. ^{252}Cf-PD mass spectra of angiotensin II + Cu(II) (1 : 1 molar ratio) adsorbed on nitrocellulose from aqueous solutions with different pH values: (a) pH = 2.5, (b) pH = 3.4, (c) pH = 5.3.

Further evidence for a correlation between solution chemistry and ^{252}Cf-PDMS of adsorbed species was obtained from studies of the 'kinetically inert' Pt(II) complexes of ang II, where the rate of complex formation at room temperature is extremely slow (days) but can be reduced in time to minutes at higher solution temperatures. The ^{252}Cf-PD mass spectrum of adsorbed species from solutions of Pt(II) + ang II reflected the slow kinetics of complex formation and sensitive dependence on temperature. Cu^{63}/Cu^{65} exchange on ang II confirmed that Cu(II) bound to ang II is kinetically labile. Thus, most of the known kinetic behavior of these complexes in aqueous solution could be monitored and confirmed by ^{252}Cf-PDMS analysis of species adsorbed from solution onto nitrocellulose.

However, the mass spectra did include ions that were formed or modified in the desorption/ionization process. For example, the Cu^+ ion, which was only observed when the Cu complex was present in the sample, is a fragment ion from Cu/ang II molecular dissociation at high excitation energy. That Cu^+ is observed and not Cu^{2+} is due to gas-phase reduction of Cu^{2+} in the desorption plume. There is also evidence for the formation of molecular ions where more than one Cu is attached. It is unlikely that these species form in solution when equal molar amounts of Cu(II) and ang II are present but these species could be formed by collisions with neutral Cu atoms in the desorption plume.

In these ang II + Cu(II) studies using the interfacial solution nitrocellulose adsorption ^{252}Cf-PDMS protocol, an observation was made that is not consistent with what is known about Cu(II) binding sites for this peptide derived from solution studies. The discrepancy may be due to differences between gas-phase and solution-phase chemistry. The N-terminal amine is the primary Cu(II) binding site at a pH of 6, and at higher pH values the His^6 residue also becomes a binding site. It is not straightforward to determine with certainty the binding site and coordination sphere for a peptide/transition metal ion complex by any solution-based technique, so the possibility of using the fragmentation patterns inherent in a ^{252}Cf-PD mass spectrum is a particularly attractive new approach. The relative intensities of the fragmentation pattern for the ang II + Cu(II) complex shown in Fig. 9 in histogram form, clearly shows the involvement of both the N-terminal and His^6 domains as primary coordination sites which then stabilize the corresponding a_2 and a_6 fragment ions resulting in an enhanced intensity relative to the native form. The His^6 coordination site was confirmed by the appearance of a significant Cu-containing

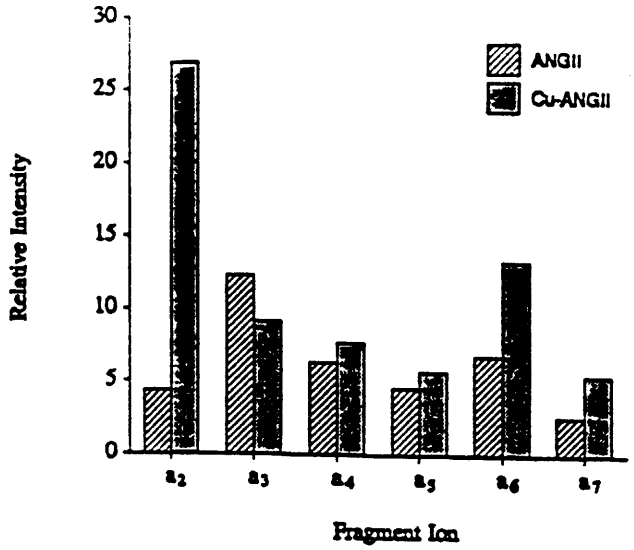

Figure 9. Comparison of the fragment ion intensities of ang II and ang II + Cu(II) relative to the molecular ion intensity. The enhanced intensities of the a_2, a_6 and a_7 fragment ions for the Cu complex suggest stabilization of these fragment ions due to Cu(II) coordination.

inner immonium His fragment ion. But, in addition, an equally intense Cu-containing Arg immonium ion was also observed. Arginine is a weak ligand for transition metal ion coordination, so the appearance of this fragment ion was not only unexpected but is inconsistent with the solution chemistry results.

The conclusion of this study is that, while it may be possible to deduce a considerable amount of the solution chemistry of peptide/transition metal ion interactions using ^{252}Cf-PDMS, some of the details might be distorted by chemistry that is occurring in the gas phase during the desorption/ionization process. The challenge of preparing a sample for ^{252}Cf-PDMS analysis from an aqueous solution in a manner that retains much of the essential spatial and chemical character of the solute in solution remains a challenge for the future that could be resolved by an approach that is discussed in the next section.

Focusing on the *chemistry* of desorption/ionization

Since the introduction of the desorption ionization methods (^{252}Cf-PDMS, 'organic' secondary ion mass spectrometry (SIMS), fast atom bombardment (FAB), MALDI) attention has been directed toward the desorption process: how the energy impulse deposited into the condensed phase triggers the ejection of species into the gas phase. In the early stages of the development of ^{252}Cf-PDMS, 'organic' SIMS and FAB, Cooks proposed that ion formation takes place via gas-phase processes ('chemistry in the selvedge').[18] This model was not universally accepted primarily because it ignored contributions from surface-induced ionization processes. With the introduction of MALDI, it became clear that gas-phase chemistry was responsible for producing analyte molecular ions. In fact, it is likely that gas-phase chemistry is responsible for molecular ion formation in ^{252}Cf-PDMS as well. (Cooks was right!) The designer matrix concept introduced by Jungclas is based on the notion that what happens in the ejection plume determines the spectrum of ions that emerge from the plume. So, the future of ^{252}Cf-PDMS is dependent on how clever we are in controlling the chemical composition of the plume of desorbed species. The first principle in this evolution has already been achieved with the Jungclas studies: surround the analyte molecules in the condensed phase with molecules that are relatively volatile and chemically benign and which protect the analyte molecular ions once formed from ion–molecule reactions with impurities. The second principle is possibly emerging in the laboratory of Tuszynski, where a ^{252}Cf-PDMS-based protocol is being developed for the analysis of oligosaccharides.[19] A matrix is selected that disperses the oligosaccharide molecules by breaking up the intermolecular hydrogen bonds between the sugar molecules and isolating them from each other with low-molecular-mass molecules that have a basic functionality. A mechanism is proposed where the oligosaccharide molecule is desorbed as a loosely bound complex with matrix molecules that rapidly dissociates in the gas phase, freeing the analyte molecular ion. The gas-phase basicity of the matrix molecules is selected to be strong enough to compete with OH intermolecular hydrogen bonding but not so strong as to form a complex that is difficult to dissociate. Excellent results have been obtained using 3-aminopyridine as a matrix, where matrix/analyte ratios from 1:1 up to 1000:1 can be employed with a reduction in molecular ion intensity to 14% of its original value.

The future of ^{252}Cf-PDMS is very much dependent on improving molecular ion yields for large biopolymers. Presently, only one in 10 to 100 fission fragment interactions produces a molecular ion of a peptide, even though more than 1000 peptide molecules are ejected from a single fission track! The peptide is already protonated in the matrix, so the fundamental question is: *why do so few of these protonated species survive the desorption process?* The answer must lie in what is happening in the ejection plume and the question is: *can we develop the chemical insight to understand and control the chemical events that occur and preserve more of these 'pre-formed ions'?* Just *one* gas-phase protein molecular ion per fission fragment track would produce an intense ^{252}Cf-PD mass spectrum for these species. A closer look at ion pair dissociation in the gas phase—a matrix that rapidly vibrationally cools the molecular ions in the plume—may be the answer, but it is now clear that gas-phase chemistry is the key issue on which to focus more attention.

Expanding the *in situ* chemistry methodology

The standard method of preparing samples for ^{252}Cf-PDMS analysis involves adsorbing the analyte from a solution onto a suitable substrate. This method was first introduced by Jordan *et al.*[20] and later utilized by the Swedish group in the development of the highly effective nitrocellulose matrix.[21] The essential feature of this

method of sample preparation is that the analyte is immobilized on a surface and can be physically or chemically modified in a manner that can be monitored by sequential ^{252}Cf-PDMS analyses as demonstrated by Chait *et al.*[22] This methodology is continuing to advance with the development of new matrices and variations of the surface chemistry.

The ^{252}Cf-PDMS group at Vienna has made important contributions to the development of surface chemistry for the analysis of lipophilic species. They introduced a new matrix, 3-(3-pyridyl)acrylic acid (PAA), which has the feature that lipophilic species can be adsorbed on the surface of this matrix from nonpolar solvents such as chloroform.[23] This group developed a novel protocol for the analysis of glycolipids which suggests a new direction in this methodology. An example of a typical scenario is as follows. First, a crude extract of the class of compounds to be studied (e.g. cerebrosides, gangliosides, from brain tissue) is adsorbed onto the PAA matrix. A ^{252}Cf-PDMS measurement is made; the poor quality of the spectrum suggests the presence of impurities that quench molecular ion formation. The molecules of interest are known to be on the surface of the PAA but they are not yet in a form that will produce gas-phase molecular ions in the desorption plume. The presence of ion-quenching impurities is suspected. A series of *in situ* clean-up steps are performed using the same sample by flooding the surface of the PAA containing the immobilized analyte molecules with an array of solvents selected to remove impurities but not the analyte molecules. The ^{252}Cf-PD mass spectrum gives an indication that the concentration of impurities has been depleted but the molecular ion intensities of the analyte molecules are weak, indicating that a favorable reaction pathway is still not available for forming molecular ions. Based on the knowledge that the analyte molecules possess nucleophilic domains, the immobilized analyte molecules are then treated with saturated aqueous NaCl to provide Na^+ ions for gas-phase ion–molecule reactions. The sample is then reanalyzed by ^{252}Cf-PDMS and now $[M + Na]^+$ ions of the analyte molecules are observed in high yield. Molecular masses are then determined. Further experiments with the *same* sample indicate that the negative ion spectra contain structurally significant fragment ions but their intensities are suppressed by the presence of Na^+. After the molecular mass determination has been made, the PAA surface containing the sodiated analyte molecules is washed with water. The Na^+ ions are removed, the positive molecular ion spectrum is diminished but now the negative ion fragmentation pattern is enhanced. Being able to use the same sample in a sequence of chemical and physical manipulations is a feature that is particularly attractive when only small amounts of natural product analytes are available. Even after a complex sequence of operations are carried out, the analyte molecules can be recovered from the matrix for continuing studies. An example of this protocol is portrayed in Fig. 10, where a natural mixture of lactocerebrosides isolated from bovine brain is analyzed. Figure 10(a) shows the negative ion spectrum and Fig. 10(b) the positive ion spectrum after treating the adsorbed layer with saturated NaCl.

One of the most intriguing variations of the *in situ* methodology that will clearly be a component of future studies using ^{252}Cf-PDMS represents an excursion from the use of ^{252}Cf-PDMS for molecular mass/structure characterization to the chemical kinetics arena. The interaction of immobilized molecules with other species can take place at a solution/surface interface in a timed sequence. To illustrate the principle, the kinetics of the interaction of Cu(II) with angiotensin II immobilized on nitrocellulose has been studied. First, a set of identical ang II/nitrocellulose samples is prepared. A droplet of aqueous Cu^{2+} is placed on the surface of each target for a variable period of time. After a preset time, the reaction is quenched by rapid removal of the droplet. Figure 11 shows the results of this study. With a 5 s exposure (Fig. 11a), little coordination of Cu(II) has occurred; after 1 min, the MCu^+ ion intensity has increased by a factor of 3 and after 20 min it becomes the dominant molecular ion. The conclusion of this study was that it is quite feasible to study the kinetics of transition metal ion–peptide interactions by this technique. It was also possible to demonstrate that competitive coordination reactions can be studied using *in situ* chemistry. Using the Cu(II)/ang II sample prepared with the 20 min solution exposure, a droplet of aqueous ethylenediaminetetraacetic acid (EDTA) was deposited on the surface for 1 min and spun off. The Cu(II) was completely removed from the peptide because the binding constant to EDTA is much higher than for the peptide and the mass spectrum returned to that of the native peptide.

Carrying out chemical reactions at a surface/solution interface and monitoring these reactions in a sequential fashion using the essentially non-destructive feature of ^{252}Cf-PDMS suggests uses of this methodology to mimic *in vivo* cell surface-mediated processes. The first challenge would be to develop a collection of synthetic thin film matrices that mimic the structure/function of a particular cell surface (e.g. a lymphocyte, ventricular myocyte or hepatocyte). The binding of the target molecule and its conversion to a metabolite on the surface of the cell mimic could be monitored in a time sequence of ^{252}Cf-PDMS measurements. An illustration of how this protocol might be used to contribute to the solution of a problem in medicine comes from a recent study relating to adverse reactions associated with the co-administration of an antihistamine, terfenadine, with an antifungal agent, ketoconazole. Patients who are taking both drugs are at higher risk for cardiac arrhythmia, which is sometimes of sufficient intensity as to induce cardiac arrest. Terfenadine is metabolized to the active form by hepatic oxidation (aliphatic methyl group to carboxyl) in hepatocytes containing the cytochrome P-450 3A4 domain. The carboxylated metabolite circulates in the bloodstream and participates in antihistaminic activity. Ketoconazole competes with terfenadine at the active sites of the hepatocyte and inhibits the oxidation of terfenadine. The result is an elevated level of plasma terfenadine. Unfortunately, terfenadine blocks the K^+ transport channels in ventricular myocytes and this action is the cause of the disruption of the activity of the venticular electrical circuitry.

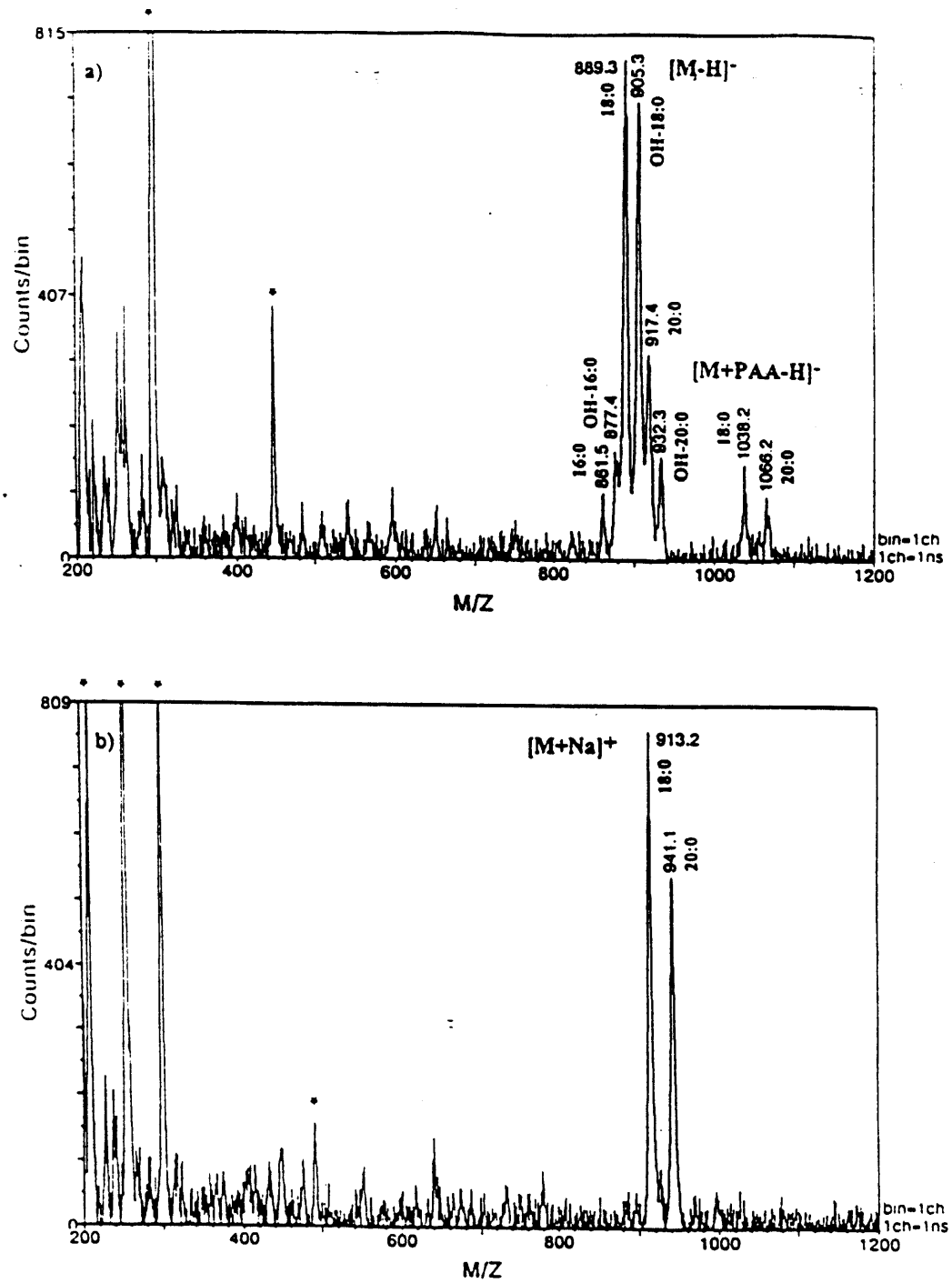

Figure 10. ^{252}Cf-PD mass spectra of a natural mixture of lactocerebrosides isolated from bovine brain demonstrating the utilization of *in situ* surface modification. The negative ion spectrum (a) was obtained after extensive washing of the analyte adsorbed on 3-(3-pyridyl) acrylic acid. The positive ion spectrum (b) was obtained after the same sample was treated with aqueous NaCl.

To simulate the hepatic oxidation of terfenadine *in vivo* a thin film of a phospholipid bilayer could be prepared that would simulate the background support structure of the hepatocyte. Cytochrome P-450 or a synthetic mimic with similar redox activity could then be incorporated into the phospholipid film. The terfenadine oxidation could be monitored by studying the reaction at the solution/film interface using ^{252}Cf-PDMS. The influence of the presence of ketoconazole in the solution on the kinetics of the oxidation could then be studied. Using a similar protocol, a mimic of the surface of a venticular myocyte containing a K^+ channel, (e.g. α-cyclodextrin) might be used to study the inhibition of K^+ complexation in the presence of terfenadine. The main advantage of ^{252}Cf-PDMS for this study is the high specificity of mass spectrometry in general in identifying substrates and metabolites by molecular mass as well as high sensitivity for the surface

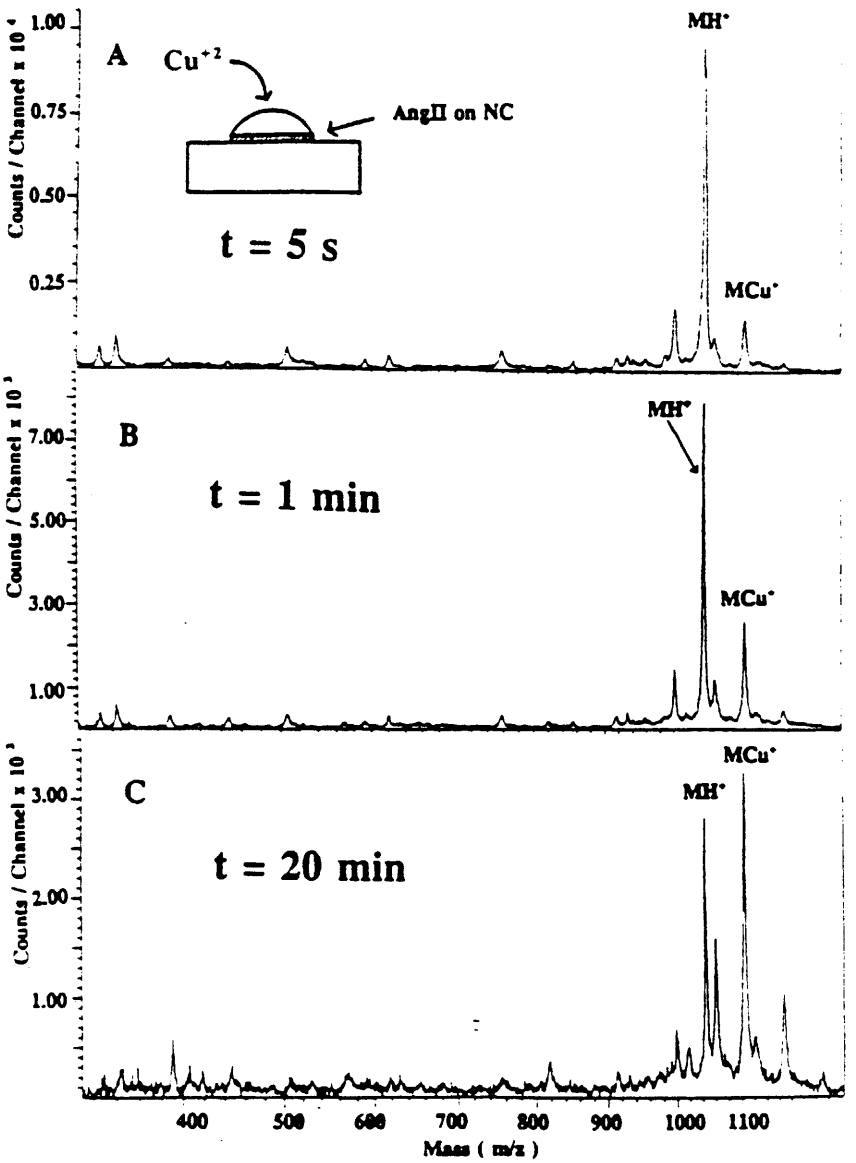

Figure 11. Kinetics of the reaction of aqueous Cu^{2+} with ang(II) molecules adsorbed on nitrocellulose for different solution exposure times (a) 5 s, (b) 1 min, (c) 20 min.

composition of the matrix. In addition, the ability to carry out serial measurements using the same matrix is an attractive feature of the ^{252}Cf-PDMS method.

SUMMARY

The ^{252}Cf-PDMS method is a 'mature' technique, having survived 20 years of research, development and application. Recent advances in the application of the method have led to the use of the technique in clinical quantitation studies, the development of a microscopic method for measuring chemical homogeneity, *in situ* applications where serial modifications can be made and studied in a sequential fashion and the utilization of the primary fragmentation patterns to correlate with primary structure. Future directions in research, development and application will focus more attention on the chemistry of the ^{252}Cf-PDMS process and the understanding and control of gas-phase reactions that occur in the ejection plume. Predictions are that the application of the *in situ* modification technique could lead to a new approach to pharmacokinetic studies as well as cell surface interactions where ^{252}Cf-PDMS is effectively used to expand the capabilities of *in vitro* studies of biological processes.

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REFERENCES

1. B. T. Chait, B. F. Gisin and F. H. Field, *J. Am. Chem. Soc.* **104**, 5157 (1982).
2. H. J. Vorst, M. W. E. M. van Tilborg, P. A. van Veelen, U. R. Tjaden and J. van der Greef, *Rapid Commun. Mass Spectrom.* **4**, 202 (1990).
3. D. M. Bunk and R. D. Macfarlane, *Proc. Natl Acad. Sci. USA* **89**, 6215 (1992).
4. K. B. Tomer, F. W. Crow and M. L. Gross, *J. Am. Chem. Soc.* **105**, 5487 (1983).
5. D. M. Bunk and R. D. Macfarlane, *J. Am. Soc. Mass Spectrom.* (in press) (1994).
6. D. Suckau, J. Kohl, G. Karwath, K. Schneider, M. Casaretto, D. B.-Suermann, and M. Przybylski, *Proc. Natl Acad. Sci. USA* **87**, 9848 (1990).
7. H. Jungclas, K.-H. Pflüger, L. Schmidt and M. Hahn, *J. Physique, Colloque C2* (Suppl. to no. 2) **50**, C2-41 (1989).
8. H. Jungclas, L. Schmidt, P. Köhl and H.-W. Fritsch, *Int. J. Mass Spectrom. Ion Proc.* **126**, 157 (1993).
9. M. A. Park, K. A. Gibson, L. Quinones and E. A. Schweikert, *Science* **248**, 988 (1990).
10. B. D. Cox, M. A. Park, R. G. Kaercher and E. A. Schweikert, *Anal. Chem.* **64**, 843 (1992).
11. M. Salehpour, P. Håkansson, B. Sundqvist and S. Widdiyasekera, *Nucl. Instrum. Methods* **B13**, 278 (1986).
12. S. Della Negra, D. Jacquet, I. Lorthiois and Y. LeBeyec, *Int. J. Mass Spectrom. Ion Phys.* **53**, 215 (1983).
13. M. Karas and F. Hillenkamp, *Anal. Chem.* **60**, 2299 (1988).
14. C. M. Whitehouse, R. N. Dreyer, M. Yamashita and J. B. Fenn, *Anal. Chem.* **57**, 675 (1985).
15. R. D. Smith and K. J. Light-Wahl, *Biol. Mass Spectrom.* **22**, 493 (1993).
16. B. D. Reverend, F. Liman, C. Livera, L. D. Pettit, S. Pyburn and H. Kozlowski, *J. Chem. Soc. Dalton Trans.* 887 (1988).
17. Z. Hu, PhD dissertation, Texas A&M University (1993).
18. R. G. Cooks and K. L. Busch, *Int. J. Mass Spectrom. Ion Phys.* **53**, 111 (1983).
19. J. O. Metzger, R. Woisch, W. Tuszynski, R. Angermann and J. Puls, *Rapid Commun. Mass Spectrom.* **7**, 1041 (1993).
20. E. A. Jordan, R. D. Macfarlane, C. R. Martin and C. J. McNeal, *Int. J. Mass Spectrom. Ion Phys.* **53**, 345 (1983).
21. G. P. Jonsson, A. B. Heddin, P. Håkannson, B. U. R. Sundqvist, B. G. Säve, P. F. Nielsen, P. Roepstorff, K. E. Johansson, I. Kamensky and M. S. L. Lindberg, *Anal. Chem.* **58**, 1084 (1986).
22. B. T. Chait and F. H. Field, *J. Am. Chem. Soc.* **107**, 6743 (1985).
23. G. Allmaier, E. Pittenauer and E. R. Schmid, *Org. Mass Spectrom.* **26**, 804 (1991).