Small Molecules as Substrates for Adsorption/Desorption in ^{252}Cf Plasma Desorption Mass Spectrometry

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We present a new approach to substrate selection for californium-252 plasma desorption mass spectrometry (^{252}Cf -PDMS) in which small volatile molecules that are water insoluble are used as matrices in place of the polymeric substrates used in previous studies. The desirable features of analyte adsorption are combined with the concept of using a volatile matrix to reduce the level of internal excitation of a desorbed analyte and to assist in ionization during the desorption process. Derivatives of anthracene were found to meet these requirements and to perform satisfactorily as substrates in ^{252}Cf -PDMS. Spectra were obtained for bovine insulin (m/z 5734) adsorbed onto 9-anthroic acid and 2-aminoanthracene and compared with spectra using a nitrocellulose substrate. Sharper peaks and lower backgrounds are observed when the 9-anthroic acid matrix is used, indicating reduced levels of internal excitation and initial kinetic energy for the desorbed molecular ion of insulin. A comparison of the performance of 9-anthroic acid and 2-aminoanthracene shows the influence of substrate functional groups on desorbed protein yields. Finally, the versatility of the small-molecule matrix concept is discussed with respect to selection of a range of functionality, solubility, and hydrophilicity. (*J Am Soc Mass Spectrom* 1991, 2, 29–32)

We report here a new approach to the selection of substrates for californium-252 plasma desorption mass spectrometry (^{252}Cf -PDMS) that retains the attractive features of analyte adsorption from aqueous solutions while providing flexibility in terms of functionality and solvent compatibility. The new strategy is that the polymeric substrates used in previous studies are replaced by matrices consisting of small volatile molecules.

The method of ^{252}Cf -PDMS is based on the excitation of solid matrices by ^{252}Cf fission fragments, which results in the desorption of molecular ions. This technique is particularly useful for mass spectrometry of high molecular weight, involatile peptides [1]. Biomolecules with molecular weights up to 45,000 have been successfully detected by using ^{252}Cf -PDMS [2]. Sample preparation is an important factor in the attainment of quality spectra from this method. In early PDMS experiments, solid matrices were prepared by evaporating solution onto a metallic foil backing. This was replaced by the electrospray technique, in which a multilayer deposit of the sample is created [3]. Efficient formation of gas-phase molecular ions from such matrices is limited by the many complex interactions taking place between molecules in the matrix during the desorption process. As a result, the presence of a high level of impurities in the matrix

can quench the ions of interest in the spectrum. In addition, many of the desorbed molecular ions can undergo metastable decay if the internal excitation is high, which contributes to peak broadening and background in PDMS spectra [4]. To reduce the intermolecular forces between the molecules of interest and to gain some control over the environment of these molecules, Jordan et al. [5] introduced the idea of adsorbing the biomolecules onto an electrosprayed matrix. Nafion [5], polyethylene terephthalate (Mylar) [6], and nitrocellulose [7] have been used as matrices in PDMS. Proteins adsorbed onto nitrocellulose adhere so strongly that salts and impurities can be rinsed from the substrate. This important feature provides a means for purifying the immobilized analyte so that impurity quenching is reduced. In addition, Chait [8] showed that ions desorbed from nitrocellulose contain less excitation energy and as a result show much less decomposition than those desorbed from electrosprayed multilayer deposits.

Recently, Karas and Hillenkamp [9] showed that protein molecular ions are photodesorbed in high yields from matrices composed of small molecules, specifically nicotinic acid. These matrices, when coupled with laser excitation, have made possible the detection of proteins up to m/z 175,000 [10]. The sample is prepared by dissolving the molecules of interest in aqueous nicotinic acid and subsequently forming a heterogeneous crystalline matrix. The protein molecular ions produced in this process are

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formed with low internal excitation. The volatility of the nicotinic acid may be a factor in the desorption process, providing a localized high-density gas containing predominantly molecules of nicotinic acid along with a low concentration of protein molecules and oligomers. Although it is yet to be established, the collisional interactions between the matrix and protein molecules may provide a means for maintaining a low level of internal excitation of the protein as well as contributing to ionization by proton transfer. The basic concept of using a volatile matrix to influence the internal excitation and ionization of an involatile analyte is an important contribution to the particle-induced desorption methodologies.

The primary objective of this study is to determine whether similar effects might also be observed for ^{252}Cf -PDMS with a suitable volatile matrix. In a search for such a matrix, we added an additional constraint: that the analyte should be adsorbed onto the matrix from solution and immobilized in a manner similar to adsorption onto nitrocellulose. This would mean that the adsorbed layer could be subsequently modified by rinsing to remove undesirable salts and by *in situ* chemical reactions such as enzymatic cleavage. We have discovered that a class of small functionalized aromatic compounds which are volatile and water insoluble possess these features.

Experimental

The substrates used in these experiments were fused-ring aromatics that are derivatives of anthracene. Nitrocellulose was included in the study for comparison. The substrates were dissolved in acetone (1 $\mu\text{g}/\text{L}$) and electrosprayed onto 1.5- μm thick aluminized Mylar films. Details of the electrospray procedure have been described previously [3], but a small modification was made to the established procedure. Scanning electron micrographs taken of our electrosprayed films showed that when the established procedure is used, the deposits tend to be thicker in the center and thinner at the edges. In addition, the particulate size is much smaller at the edges of the sprayed area than in the center. We discovered that the smaller particulates could be uniformly distributed over the target film by changing the location of the electrospray needle relative to the film. The electrospray needle was positioned at a greater distance above the film (approximately 3.5 cm) than in the past to obtain a larger spray region. In addition, the needle was displaced 7.5 mm off axis from the center of the 1.8-cm diameter target. A 300- μL volume of substrate solution was sprayed onto a collimation plate with a 1.25-cm diameter aperture centered over the target film. The collimation plate and film were slowly rotated during the spraying process. Under these conditions, the finer mist at the edge of the spray was distributed onto the film while the less uniform component was collected on the collimation plate. As a result, more uniform,

reproducible, and finer particulate matrices were obtained. Scanning electron micrographs of these electrosprayed films showed that the average particulate size of the deposit is 1 μm . The electrosprayed substrates were 1.25 cm in diameter and estimated by alpha particle energy degradation to be 100 $\mu\text{g}/\text{cm}^2$ thick.

Bovine insulin (m/z 5734) (Serva Biochemicals) was dissolved in an aqueous solution of trifluoroacetic acid (0.1%) to give a 10^{-3} M concentration. A 2- μL volume of solution was deposited onto each matrix and covered with a 12-mm diameter cover glass to spread solution over the substrate surface. Adsorption times were 4 min, followed by rinsing with 150 μL of water and spinning dry with a photoresist spinner (Headway Research, Garland, TX) at 10,000 rpm for 30 s. The rinsing step was repeated, allowing the water to remain on the surface for 30 s before spin drying.

The basic details of the spectrometer used in this study have been described elsewhere [11]. The linear configuration employed includes an ion flight path length of 53 cm. Positive ions were accelerated to ± 15 kV, while negative ions had -10 kV acceleration applied. The effective fission fragment intensity was 1500/s. All spectra were acquired for 30 min.

Results and Discussion

The derivatives of anthracene were chosen as substrates for this study according to specific criteria. First, they had to be soluble in organic solutions (e.g., acetone and ethanol) so that they could be electrosprayed. Second, only those molecules insoluble in water were selected so that proteins could be adsorbed from aqueous solutions onto the surface of the substrate in the same manner as with a nitrocellulose substrate. Third, the substrate/adsorbate matrix was required to be stable through repeated rinsing cycles. The fourth criterion was that the small molecules undergo sublimation as does nicotinic acid in the laser desorption studies. Several derivatives of anthracene were found that met these criteria and gave high-quality mass spectra by ^{252}Cf -PDMS. In this article, we report results for two of these derivatives, 9-anthroic acid ($\text{C}_{14}\text{H}_9\text{COOH}$) and 2-aminoanthracene ($\text{C}_{14}\text{H}_9\text{NH}_2$), to demonstrate the influence that substrate chemical functionality has in applications to ^{252}Cf -PDMS.

Positive and negative ion spectra of insulin from each substrate are compared in Figures 1 and 2, respectively. The molecular ion regions are shown without background subtraction. The peak height for the $[\text{M} + \text{H}]^+$ molecular ion of insulin is somewhat greater for the 9-anthroic acid matrix (Figure 1a) than for nitrocellulose (Figure 1c). More striking is the sharpness of the peaks from the 9-anthroic acid substrate along with a large reduction in background as compared with nitrocellulose. The background level using the 9-anthroic acid substrate is a factor of two less

Figure 1. Molecular ion regions for positive ion spectra of insulin on three different matrices: (a) 9-anthroic acid, (b) 2-aminoanthracene, and (c) nitrocellulose.

than in the nitrocellulose spectrum. The small unlabeled peak in Figure 1a, 222 u greater than the insulin molecular ion, corresponds to the attachment of a 9-anthroic acid molecule (m/z 222) to insulin. The negative ion spectrum of insulin from 9-anthroic acid (Figure 2a) is considerably more intense than that obtained from nitrocellulose (Figure 2c). Of particular interest is the appearance of the $[M - 2H]^{2-}$ peak in the spectrum, which had not previously been ob-

Figure 2. Molecular ion regions for negative ion spectra of insulin on three different matrices: (a) 9-anthroic acid, (b) 2-aminoanthracene, and (c) nitrocellulose.

Figure 3. Expanded view of the molecular ion peak of insulin on two different matrices: (a) 9-anthroic acid and (b) nitrocellulose.

served. A preliminary account of our new finding was given at the 1989 meeting of the American Society for Mass Spectrometry [12]. Subsequently, Beavis and Chait [13] reported the appearance of the $[M - 2H]^{2-}$ peak for an insulin spectrum recorded by using matrix-assisted laser desorption mass spectrometry.

Figure 3 shows an expanded portion of the insulin $[M + H]^+$ region for the 9-anthroic acid (a) and nitrocellulose (b) matrices. Not only is the peak width (FWHM) less for the 9-anthroic acid matrix, but also the low-mass structure of the peak is greatly reduced. This component corresponds to fragments of molecular ions that have undergone prompt fragmentation prior to acceleration or metastable decays in the field-free region of the spectrometer [4]. The reduction in the metastable component along with the surprising reduction in peak width is an indication that the insulin molecular ions are desorbed from the anthroic acid matrix with less internal energy than from nitrocellulose. The reduction of the background, which has a large metastable ion and neutral component, is further evidence that the anthroic acid substrate is providing some vibrational cooling for the insulin molecular ion. The desorbed insulin may have less internal energy because of the mechanism of desorption from this matrix. The appearance of the insulin-anthroic acid adduct ion in the spectrum proves that there is a significant interaction between insulin and anthroic acid, probably through hydrogen bonding at the basic sites on insulin. It is likely that insulin encounters several anthroic acid molecules during the desorption process, through hydrogen bonding when insulin is adsorbed on the surface and through collisions when the insulin and surrounding molecules are desorbed from the surface. Through these interactions, energy relaxation and ion formation of insulin might occur.

The molecule 2-aminoanthracene was chosen for comparison with 9-anthroic acid to study the influence on desorption and ionization of the substrate functional group. As shown in Figures 1b and 2b, the

molecular ion yield is drastically reduced. The background of the 2-aminoanthracene matrix is comparable to that of 9-anthroic acid, and about half that of nitrocellulose in the positive-ion spectra. The large difference in the behavior of 9-anthroic acid and 2-aminoanthracene suggests that the carboxyl functional group is indeed playing an important role in the protonation of insulin.

As discussed earlier, both the positive and negative mass spectra of insulin using the 9-anthroic acid substrate (Figures 1a, 2a, and 3a) exhibit much sharper peaks than are observed using a nitrocellulose matrix, and this is partly attributed to a reduced level of metastable ion formation. The existence of the metastable ion component in the molecular ion peak of insulin desorbed from nitrocellulose has recently been demonstrated by using electrostatic ion retardation techniques [14]. In the past, we showed that the initial kinetic energy distribution of the desorbed molecules contributes to peak broadening in the spectrum. We also showed that for molecules desorbed from a nitrocellulose substrate, surface charging is responsible for a large increase in the initial kinetic energy distribution [15]. The fact that anthracene is photoconductive [16] suggests that the presence of charge carriers with high mobility in the vicinity of the fission fragment track in the anthracene derivative substrate could decrease the amount of surface charging surrounding the fission fragment track.

Conclusions

In summary, we have shown that 9-anthroic acid, a small molecule that is water insoluble and volatile, can be successfully used as a matrix for ^{252}Cf -PDMS in a manner that polymeric matrices have been used in the past. When 9-anthroic acid is used as a substrate for proteins, sharp peaks are observed in the mass spectrum that indicate a reduction of internal excitation and initial kinetic energy spread. Although we have focused our discussion on results for anthroic acid, other small molecules have also given good-quality protein spectra in our laboratory. These include anthrarobin, purpurogallin, purpurin, alizarin, quinalizarin, and anthraquinone-2-carboxylic acid. Finally, the use of small molecules as substrates has advantages over the use of polymeric materials, such as nitrocellulose, while maintaining the ability to use adsorption and a solid-solution interface to prepare samples. The functionality, solubility, hy-

drophilicity, and volatility of the substrate surface can be easily modified by selecting molecules that have the desired characteristics. Mixed functionality can be introduced into the surface of the substrate by using mixtures of small molecules that have the desired functionalities. For example, a substrate with a high concentration of carboxyl and amino groups can be prepared by electro spraying a mixture of 9-anthroic acid and 2-aminoanthracene.

One of the problems in the past in substrate development for ^{252}Cf -PDMS applications based on polymeric materials has been the difficulty in modifying the functionality of the polymer and achieving a high concentration of functional groups on the surface. The introduction of the small-molecule matrix concept makes it possible to study the role of the substrate in ^{252}Cf -PDMS in a more controlled manner.

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