Effects of Protein−Surface Interactions on Protein Ion Signals in MALDI Mass Spectrometry

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Effects of Protein–Surface Interactions on Protein Ion Signals in MALDI Mass Spectrometry

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The influence of polymer surface–protein binding affinity on protein ion signals in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is examined. The surfaces of poly(vinylidene fluoride) and poly(ethylene terephthalate) polymer substrates are modified by pulsed rf plasma deposition of allylamine. By varying the on/off duty cycle of the pulsed rf plasma, the polymer substrate surfaces are coated with thin films having varying densities of surface amine groups. The varying surface amine density is shown to lead to systematic changes in the surface binding affinity for the ^{125}I-radiolabeled peptides angiotensin I and porcine insulin. Unlabeled angiotensin I and porcine insulin are then deposited on the pulsed rf plasma-modified substrates and analyzed by MALDI mass spectrometry. The experimental approach involves applying the peptide to the modified polymer surface in an aqueous phosphate-buffered saline solution and allowing the peptide solution to dry completely under ambient conditions. Subsequently, the MALDI matrix α-cyano-4-hydroxycinnamic acid in methanol and 10% trifluoroacetic acid in water are added to the peptide-coated modified polymer surfaces. The results of these studies demonstrate that, for the sample preparation method employed, increases in the surface peptide binding affinity lead to decreases in the peptide MALDI ion signal.

In recent years, matrix-assisted laser desorption/ionization (MALDI)[1] mass spectrometry has been combined with a wide variety of bioanalytical techniques, many of which require the sample to be desorbed from surfaces other than a standard metal target. For example, MALDI mass analysis has been coupled with both one and two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).[2] In these applications, MALDI of the gel-separated proteins is generally performed after electroblotting the proteins from the gel onto a polymer membrane. A protein-containing band of the polymer is cut from the membrane, affixed to a metallic MALDI sample probe, coated with the MALDI matrix of choice, and mass analyzed by conventional methods. In an extension of this approach, MALDI instruments have been modified so that the proteins distributed along the entire membrane can be analyzed by progressively stepping the matrix-coated membrane through the source region of the mass spectrometer.

A variety of membranes have been evaluated for the applications described including poly(vinylidene fluoride) (PVDF),[3−7] nylon,[8] polyethylene (PE),[9] and polyurethane (PU).[10] In these reports, attention has been given to the influence of the membrane choice and other MALDI experimental parameters (wavelength, irradiance, matrix solvent, etc.) on the analytical utility of the protein MALDI mass spectra. Protein ion signal intensity, ion signal resolution, and the ability to obtain analytically useful MALDI mass spectra from high-mass proteins have all been shown to be strongly influenced by the choice of polymer substrate. Several of these studies have attributed the observed effects to morphological features (pore size and heterogeneity) of the polymer substrate.[5,9] For example, high-porosity PVDF membranes were shown to be less desirable as polymer substrates due to penetration of the matrix/analyte crystals into the bulk polymer membrane, resulting in less efficient sample desorption by the UV laser pulse.

A number of these studies have also suggested that adsorption of the protein to the polymer membrane might lead to reduced protein MALDI ion signals.[6,8] This effect is expected to interfere with the cocrystallization of the MALDI matrix and the protein. On the other hand, protein–polymer substrate adsorption has been cited as an advantage in cases where these interactions are thought to allow residual salts and surfactants to be washed from the polymer membrane without loss of protein.[8,10,11] In at least one report, it has been recognized that a careful balance between these two effects would characterize the ideal membrane for

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MALDI of SDS−PAGE-separated proteins; i.e., adsorption of the protein to the polymer membrane must be strong enough to allow manipulation of the sample, but weak enough to be overcome during the matrix deposition step.[8] Despite these qualitative observations, no systematic study of the influence of protein−polymer substrate adsorption on MALDI ion signals has been performed.

The production of polymer substrates with varying affinities for protein binding has been the subject of considerable research in the field of biomedical engineering. To this end, plasma modification of polymer surfaces has been employed to alter both the nature and density of the polymer surface chemical functionality. For example, extensive studies have been conducted using continuous wave (CW) plasma polymerizations of fluorocarbons to modify surfaces for subsequent measurements of protein−surface binding interactions.[12−14] More recently, we have employed variable duty cycle pulsed rf plasmas to control the density of surface chemical functionalities while maintaining the integrity of the deposited polymer film. These surfaces have been shown to allow the production of surfaces with varying affinity for protein adsorption through the control of the density of the surface chemical functionalities.[15,18]

In the present studies, we have systematically investigated the influence of increasing polymer surface−protein binding affinities on the protein MALDI ion signal intensities. These studies are performed by modifying the polymer surface−protein binding affinity by pulsed rf plasma deposition of allylamine. The influence of these modifications on the surface−protein binding affinity is confirmed by conventional radiolabeled protein binding studies. Our results demonstrate that alterations of the polymer surface that lead to increases in the surface−protein binding affinity lead to corresponding decreases in the protein MALDI ion yield. The results obtained in the present work are of significant importance to present and future studies where MALDI mass spectrometric methods are used to quantitate surface-bound proteins.

EXPERIMENTAL SECTION

Chemicals and Materials. The materials PVDF (0.25-mm thickness) and poly(ethylene terephthalate) (PET, 0.1250-mm thickness) were obtained from Goodfellow Corp. (Berwyn, PA). Allylamine was purchased from Aldrich Chemical Co. Phosphate-buffered saline (PBS) was purchased from Sigma and prepared as directed. Angiotensin I, porcine insulin, and the MALDI matrix, α-cyano-4-hydroxycinnamic acid (ACHCA) were purchased from Sigma and used without further purification. ^{125}I-Labeled peptides were provided by the University of Texas Southwestern Medical Center (Dallas, TX). The 0.3% SDS (Sigma) solution and the 10% (v/v) trifluoroacetic acid (TFA, Sigma) solution were prepared by dilution of the pure substances with distilled water.

Pulsed rf Plasma Modification and Surface Analysis. The pulsed rf plasma polymerization technique and apparatus employed to modify the surfaces of the polymeric substrates have been described previously.[16,17] In the present study, the PVDF and PET substrates (either 0.5-cm-diameter disks or 1.0×2.0 cm rectangular pieces) were cleaned ultrasonically and then placed in the center of a 10.0×30.0 cm cylindrical glass reactor. The substrates were initially treated with a 10-min pulsed argon plasma to provide additional surface cleaning. Subsequently, the Ar flow was terminated and the entire system was evacuated to background pressure (10 mTorr). The allylamine monomer was then introduced at a flow velocity of ∼4 cm³/min. (STP), giving a reactor pressure of 70 mTorr under nonplasma conditions.

The polymerization of the allylamine was carried out at a constant power input of 200 W using three different plasma duty cycles (plasma on/plasma off times in ms) namely: 3/5, 3/15, and 3/45. Previous work in our laboratory has demonstrated that a significant variation in the composition of the polymeric allylamine films is observed over this range of duty cycles.[18,19] Although these variations produced relatively small increased nitrogen atom content in the films with decreasing duty cycle, large variations in N atom chemical functionalities are observed. This latter variation included particularly large increases in the surface density of amine groups as the average power input (i.e. plasma duty cycle × peak power) during polymerization is reduced. The duty cycle dependence of the amine groups was established via the standard reaction of the plasma polymer allylamine films with gaseous trifluoroacetic anhydride[20,21] followed by subsequent analysis of the surface fluorine atom incorporation using XPS analysis.[22]

XPS analysis of the pulsed rf plasma-modified surfaces was performed using a Perkin-Elmer PSI 5000 series instrument. The Al Kα X-ray source at 1486.8 eV impinged on the pulsed rf plasma-modified polymer surfaces at a 70° angle relative to the sample surface. Charge buildup on the polymer surfaces was neutralized using an electron flood gun during recording of the XPS spectra. Calibration of the XPS spectra was performed by centering the lowest binding peak of the C(1s) multiplet at 284.6 eV.[23] Reported carbon, nitrogen, and oxygen atom percents are the average of three separate runs and have uncertainties of ∼±2%.

[125]I-Radiolabeled Peptide Adsorption. Quantitation of the peptide binding affinity of the pulsed rf plasma-modified PVDF and PET polymer samples was investigated by exposure of the samples to ^{125}I-radiolabeled peptides. These studies were performed in a flow-through cell to avoid denaturation of the peptides at the air−water interface and to avoid formation of Langmuir−

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Blodgett films resulting from immersion of the polymers in peptide solutions. PVDF and PET polymer samples (1.0 cm × 2.0 cm), which had been modified by variable duty cycle pulsed rf plasma deposition of allylamine, were placed in a flow-through cell with a total internal volume of 20 mL. Initially, PBS (pH 7.4) solution was introduced into the cell and the polymer samples were allowed to equilibrate for 3 min. The PBS solution was then flushed from the cell by the introduction of 30 mL of either ^{125}I-radiolabeled porcine insulin (0.010 mg/mL in PBS, 1.0 μCi/mL) or ^{125}I-radiolabeled angiotensin I (0.010 mg/mL in PBS, 1.0 μCi/mL). Incubation of the polymer samples for 20 min followed introduction of the peptide solution. Subsequently, the peptide solution was displaced by introduction of 50 mL of PBS solution. Finally, the flow-through cell was drained and the polymer samples were removed.

Adsorbed peptide was measured by placing the polymer samples in test tubes and measuring the radioactivity using a well-type γ counter (Wallac 1282, Compugamma). Quantitation of adsorbed protein was determined by comparison of the measured radioactivity with the radioactivity of 200 μL of peptide solution having a known concentration. Subsequently, 4-mL volumes of 0.3% SDS solution were added to each test tube and the samples were incubated for 3 min with intermittent shaking. The samples were then removed to fresh test tubes, and the radioactivity was recounted to determine the quantity of protein retained by the substrates. Quantitation of the retained protein was determined using the procedure described above.

MALDI Analysis. All MALDI mass spectra were acquired using a home-built linear time-of-flight (TOF) mass spectrometer in which samples are introduced through a hole in the center of the repeller electrode in the TOF source region. Laser desorption is performed using an Oriel N$_2$ laser (337 nm) operating at a repetition rate of ~3 Hz and adjusted to intensities ~10% above threshold for analyte ion formation using a variable neutral density filter. Typical desorbing laser fluences were of the order of 0.9 J/cm^2. The ions were accelerated with an +18-kV extraction voltage applied across a 1.0-cm source region before entering a ~95-cm field free drift tube. Exiting ions were subjected to an additional 4-kV postacceleration before being collected by a Z-gap microchannel plate detector (R. M. Jordan).

Sample preparation for MALDI TOF mass analysis involved the application of peptide and matrix solutions to PVDF and PET polymer disks which had been modified by variable duty cycle pulsed rf plasma deposition of allylamine as previously described. After attaching the modified polymer disks to stainless steel probe tips using commercial adhesive, 2-μL volumes of peptide in PBS solution (either porcine insulin, 0.10 mg/mL, or angiotensin I, 0.01 mg/mL) were applied and allowed to dry for ~45 min in air. A simple calculation based on the reported radius of insulin and the radius of the polymer disk suggests that uniform distribution of this quantity of the peptide would lead to a 2−3-layer coverage of the polymer surface.[24] Subsequently, 2 μL each of the MALDI matrix ACHCA (15 mg/mL in methanol) and 10% (v/v) TFA in distilled water were co-deposited on the probe tip and allowed to dry. Three samples were prepared for each analyte/surface modification combination, and 20 mass spectra were collected from the three samples.

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Table 1. XPS Analysis of the Surface Films Deposited by Variable Duty Cycle Pulsed rf Plasma Deposition of Allylamine

pulsed rf plasma duty cycle[a]	surface composition (%)		
	carbon	oxygen	nitrogen
PVDF Polymer Substrate			
3/5	78.5	1.2	20.4
3/15	77.4	1.0	21.6
3/45	73.8	1.6	24.6
PET Polymer Substrate			
3/5	77.5	1.5	21.0
3/15	77.7	1.1	21.2
3/45	72.2	1.5	26.3

[a] The numbers shown are the pulsed rf plasma "on" and plasma "off" times (in ms). See Experimental Section for further details.

All MALDI TOF spectra were signal averaged for 50 laser shots using a LeCroy 9310AM digital storage oscilloscope before being transferred to a personal computer for mass calibration and data analysis. The integrated area of the singly protonated peptide ion signal in each spectrum was determined using the Grams/386 (Galactic Industries Corp.) peak integration routine. After application of the Q-test (90% confidence) to outliers, the average and standard deviation of the integrated peak areas was calculated. A minimum of 17 individual values are included in the average integrated peak area values reported.

RESULTS AND DISCUSSION

Previous work has shown that exposure of materials to reactive gases that have been passed through a rf plasma leads to the surface deposition of a conformal, pinhole-free cross-linked film.[25] Furthermore, several recent pulsed plasma polymerization studies have shown that variations in the on/off duty cycle of the pulsed rf plasma can lead to controlled variations in the retention of the functionality of the reactive gas in the surface-deposited film.[26−28] Analysis of the variable duty cycle pulsed rf plasma allylamine surface films used in these studies by XPS (see Table 1) indicates that as the duty cycle is changed from 3/5 to 3/15 to 3/45, there is a slight increase in the retention of nitrogen in the polymers. As noted earlier, however, the increased nitrogen atom incorporation is accompanied by a significantly larger increase in the amine groups retained in the pulsed rf plasma deposited surface film.[18,22] This behavior is believed to arise from reduced destruction of the amine functionality by the plasma under the conditions where the plasma is on a lower percentage of the total time.

The increasing amine content of the pulsed rf plasma-deposited surface films has a strong influence on the affinity of the surface for peptide binding, as shown by the ^{125}I-radiolabeled peptide binding studies. Table 2 shows the quantities of [^{125}I]angiotensin I and porcine [^{125}I]insulin initially adsorbed to the surfaces and the quantities retained after washing the surfaces using a 0.3% SDS solution. As the duty cycle is changed from 3/5 to 3/15 to 3/45, both the total quantity of peptide adsorbed to the surface and the quantity of peptide retained by the surface increases.

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The increase in biomolecule adsorption with increasing surface primary amine content is a general trend and has been observed in our laboratory for a variety of other peptides and proteins. A detailed discussion of the factors that influence surface−protein interactions is beyond the scope of this report.[29] However, it is likely that both electrostatic and hydrophilic interactions are involved. Clearly, increases in the density of surface amine functional groups will increase the hydrophilicity of the polymer surface film leading to increased hydrophilic interactions with the solution peptides.

In addition, at the pH of these experiments (both during deposition of the peptide and during deposition of the matrix), the surface amine groups are presumably protonated, leading to increases in the density of surface positive charge with increases in primary amine content. During deposition of the peptide, negatively charged regions of the solution-phase peptides would be expected to interact strongly with the positively charged surfaces. Thus, electrostatic interactions might also play a role in surface−peptide binding if the interactions are retained through the brief exposure (drying time ∼5 min) to the acidic matrix/ TFA solutions. Studies performed in our laboratory using peptides with systematic variations in pI and surfaces with positive and negative solution charges suggest that both hydrophilic and electrostatic interactions play a role in the surface−peptide binding.[30]

Figures 1 and 2 show typical MALDI TOF mass spectra of angiotensin I and porcine insulin obtained using the sample preparation procedure described in the Experimental Section. In the mass spectra shown, the peptides were applied to PVDF polymer disk surfaces modified by variable duty cycle pulsed rf plasma deposition of the allylamine. Other than the duty cycle of the pulsed rf plasma deposition of the allylamine, all other sample preparation and mass spectrum acquisition parameters were identical. The data in Table 3 summarize the changes in peptide
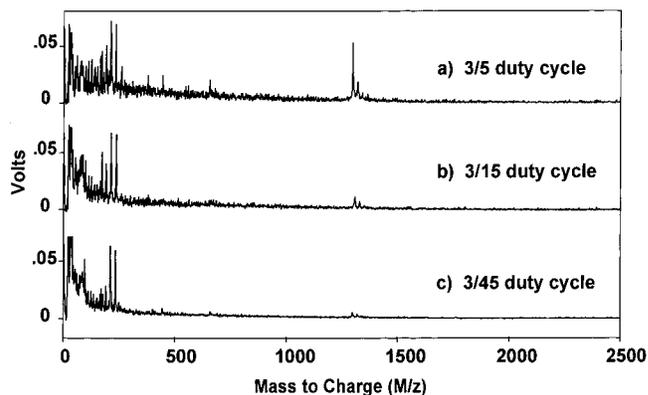
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Figure 1. MALDI TOF mass spectra of angiotensin I (MM = 1296 Da) applied to PVDF disk surfaces that have been modified by variable duty cycle pulsed rf plasma deposition of allylamine. Identical sample preparation and mass spectrum acquisition conditions have been used for each surface.
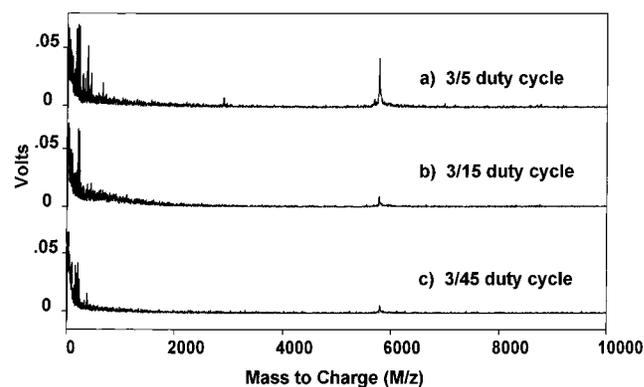

Figure 2. MALDI TOF mass spectra of porcine insulin (MM = 5778 Da) applied to PVDF disk surfaces that have been modified by variable duty cycle pulsed rf plasma deposition of allylamine. Identical sample preparation and mass spectrum acquisition conditions have been used for each surface.

MALDI ion signal with changes in pulsed rf plasma duty cycle for angiotensin I and porcine insulin applied to allylamine-modified PVDF and PET. (We note that the PVDF and PET polymers serve only as a substrate for the allylamine modification. XPS studies clearly show that the substrate is completely coated by a thin film of plasma-polymerized allylamine.) Cumulatively, the data pre-

sented clearly suggest that as the affinity of the surface for peptide binding increases, due to the increase in surface amine functionality, the magnitude of the integrated peptide MALDI ion signal decreases.

It should be pointed out that the observed inverse relationship between the magnitude of the peptide MALDI ion signal and the surface−peptide binding affinity may be a result of the specific experimental methodology used in these studies. A key feature of our sample preparation step is that the peptide solution is applied to the modified polymer disks and allowed to completely evaporate in air before small volumes of aqueous 10% TFA and MALDI matrix in methanol are applied. This methodology represents a departure from the most widely used MALDI sample preparation procedure in which both the matrix and the biomolecule analyte are co-deposited on the probe surface and allowed to cocrystallize in air.[1] In view of the ~45-min peptide solution drying time, the peptide is able to sample the primary amine-rich polymer surface to find sites of strong binding interaction. Subsequent addition of the matrix and TFA solutions may only resolubilize the less tightly bound peptides that have been unable to find high-affinity surface binding sites. Thus, less peptide is incorporated in the matrix crystals as the surface−peptide binding affinity increases. Consistent with previous studies which suggest that efficient MALDI ionization occurs for only those peptides incorporated in the crystallized matrix material,[31,32] this effect leads to reduced peptide ion signals.

Comparisons between experimental results, obtained using the PVDF and PET polymer substrates, reveal several additional points worthy of discussion. For example, as shown in Table 3, it is apparent that the integrated peptide MALDI ion signals are uniformly lower for the PVDF substrates than for the PET substrates. This decrease in peptide MALDI ion signal ranges from −4 to −72% with an average decrease of −45%. This result is mirrored by the data in Table 3 which show a consistent increase in the [^{125}I]peptide binding affinity (both adsorbed and retained) for the PVDF substrate over the PET substrate. The increase in peptide binding affinity ranges from +14 to +71% with an average increase of +33%. We have previously shown, via examination of the surfaces by atomic force microscopy,[33] that the PVDF surface is substantially rougher than the PET surface (i.e., the average rms "z" deviation for allylamine-modified PVDF and PET is 210 and 15 nm, respectively). This increase in surface roughness would clearly lead to an increase in total surface area for the PVDF substrate which could readily explain both the uniform increase in total [^{125}I]peptide binding and the concomitant uniform decrease in the peptide MALDI ion signal.

However, while there is a global increase in the MALDI ion signal when changing from PVDF to PET, consistent with a decrease in total surface area, it appears that the differentiation between the two surfaces is substantially larger for the 3/15 and 3/45 pulsed rf plasma-modified surfaces. This increased differentiation in the peptide MALDI ion signals between the PVDF and PET surfaces is not quantitatively reflected in the [^{125}I]peptide

binding studies. The explanation for this behavior is presently unclear, but may be related to a shadowing effect of the rougher PVDF surface which prevents effective laser irradiation of the crystalline matrix material in the clefts of the rougher polymer material (i.e., similar to the previously cited disadvantage of porous PVDF membranes for MALDI). If true, this effect appears to have a greater impact on the peptide MALDI ion signals when the peptides are deposited on surfaces with high peptide binding affinities. Further systematic studies of the influence of surface morphology, angle of laser irradiation, and surface chemical functionality on MALDI ion formation efficiency are presently in progress.

CONCLUSION

For the experimental conditions employed in these studies, the results clearly indicate that surface−protein binding interactions strongly influence the efficiency of protein ionization by MALDI. As the surface−protein binding affinity increases, there is a substantial consequent decrease in the protein MALDI ion signal; presumably due to poor incorporation of the protein in the crystalline MALDI matrix. The experimental methodology used in these studies is, in many ways, analogous to the methods employed for MALDI mass spectrometric analysis of electrophoretically separated proteins that have been electroblotted onto polymer surfaces. Thus, our results suggest that improved sensitivity MALDI analysis of proteins deposited on polymer surfaces could be obtained through the judicious choice of polymers with low surface−protein binding affinities and/or the use of aggressive methods for the removal of surface adsorbed proteins.

The results presented can also be viewed as indicative of a potential new application of MALDI mass spectrometry in the determination of the protein adsorption properties of biomaterials. Investigations of the influence of various material properties on protein adsorption is an area of active research with significant implications for the design of improved biomedical devices. Our results suggest that MALDI mass spectrometry could be used to rapidly assess the protein binding affinity of a new biomaterial, relative to some previously characterized material, using the approach described in this paper. Thus, MALDI mass spectrometry could be used as a rapid and efficient method to quickly assay new materials for desired increases or decreases in relative protein binding affinity prior to quantitative evaluation of the surface−protein binding affinity using conventional methods.

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