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DESIGN AND IMPLEMENTATION OF PATTERNED SURFACES FOR ON-PROBE CLEANUP AND CONCENTRATION OF PROTEINS, PROTEIN DIGESTS, AND DNA PRIOR TO ANALYSIS BY MALDI-TOF-MS

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Major Professor's Signature

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Date

DESIGN AND IMPLEMENTATION OF PATTERNED SURFACES FOR ON-PROBE CLEANUP AND CONCENTRATION OF PROTEINS, PROTEIN DIGESTS, AND DNA PRIOR TO ANALYSIS BY MALDI-TOF-MS

By

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ABSTRACT

DESIGN AND IMPLEMENTATION OF PATTERNED SURFACES FOR ON-PROBE CLEANUP AND CONCENTRATION OF PROTEINS, PROTEIN DIGESTS, AND DNA PRIOR TO ANALYSIS BY MALDI-TOF-MS

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This dissertation describes a surface science/mass spectrometry effort to develop and characterize patterned surfaces that serve as matrix-assisted laser desorption/ionization (MALDI) sample platforms capable of concentrating and purifying proteins and DNA. The use of these patterned surfaces can also enhance the detectability of peptides, especially phosphopeptides, from protein proteolytic digest mixtures. Using micro-contact printing, small (200-μm diameter) hydrophilic spots of bare gold, chemically anchored poly(acrylic acid) (PAA), or immobilized polyethylenimine (PEI) are patterned at 5-mm intervals in a hydrophobic field consisting of a self-assembled monolayer of hexadecanethiol. Dilute or salt-contaminated protein, DNA, or protein proteolytic digest samples are applied onto the hydrophilic spots, dried, and then rinsed with water to remove water-soluble contaminants, simplify a digestion mixture, or separate non-phosphopeptides from phosphopeptides. One of the key features in this work is the combination of a functionalized surface with a small spot to afford both concentration of analyte via evaporation to a small spot size and purification by selective adsorption. The polymeric anchors bind the analytes during the water-rinsing step, and the

subsequently added acidic matrix solution releases analytes for their incorporation into the matrix crystals.

Use of these patterned surfaces decreases the detection limit for the analysis of dilute protein samples by MALDI-MS. For example, 1-5 fmol of insulin chain A, insulin chain B, insulin, and ribonulcease A can be routinely observed with patterned surfaces, while conventional stainless steel probes allow only 50-100 fmol detection limits. The detection limits for salt-containing samples decrease by at least one order of magnitude for use of patterned surfaces compared with use of non-patterned on-probe decontamination methods.

The patterned surfaces also allow the detection of more tryptic peptides in protein digestion mixtures. For example, use of a patterned PAA surface revealed 22 peptides as compared to only 11 peptides observed with a SS plate. Thus, the PAA surface allows a much higher confidence level for protein identification during myoglobin peptide mapping. Patterned surfaces also allowed 13% higher sequence coverage for a larger protein, bovine serum albumin.

Modified probes containing small spots modified with polycations show great promise for selectively enriching phosphorylated peptides directly on the probe prior to MALDI-MS analysis. The positively charged anchors selectively bind the negatively charged phosphopeptides, while the water-rinse removes other signal suppressing contaminants or non-phosphopeptides.

To my loving and supportive parents

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LIST OF ABBREVIATIONS

A.....Angiotensin

AC.....Ammonium Citrate

AP.....phophorylated Angiotensin

B&B.....Bull & Breese

BSA.....Bovine Serum Albumin

α-CHCA.....α-cyano-4-hydroxy cinnamic acid

DMF.....N,N-dimethylformamide

DHB.....2,5-dihydroxybenzoic acid

DTT.....Dithiolthreitol

ESI.....Electrospray Ionization

HDT.....Hexadecanethiol

3-HPA.....3-Hydroxypicolinic Acid

HPLC.....High Performance Liquid Chromatography

IMAC.....Immobilized Metal Affinity Chromatography

MALDI.....Matrix Assisted Laser/Desorption Ionization

MLK3.....Mixed Linkage Kinase 3

MOWSE.....Molecular Weight Search

MS.....Mass Spectrometry

MUA.....Mercaptoundecanoic Acid

NaOAc.....Sodium Acetate

NTA.....Nitrilotriacetic Acid

OM.....Octadecyl Mercaptan

PAA.....Poly(acrylic acid)

PDMS.....Polydimethylsiloxane

PE.....Polyethylene

PEI.....Polyethylenimine

PP.....Polypropylene

pS.....phosphorylated Serine

pT.....phosphorylated Threonine

PTBA.....Poly(*tert*-butylacrylate)

pY.....phosphorylated Tyrosine

PU.....Polyurethane

PVDF.....Polyvinylidenedifluoride

RMS.....Root Mean Square

RNase A.....Ribonuclease A

SAM.....Self-assembled Monolayer

SS.....Stainless Steel

Teflon.....Poly(tetrafluoroethylene)

Zitax.....Poly(tetrafluoroethylene)

Chapter One: Introduction

Mass spectrometry has been an important tool for structural analysis of small molecules for a long time, but historically it has not been very useful for biochemical analyses because the ionization of non-volatile macromolecules, such as proteins and DNA, via traditional methods is difficult, if not impossible. However, the introduction of two new ionization techniques, electrospray ionization (ESI)[1] and matrix-assisted laser/desorption ionization (MALDI)[2], changed this situation. Proteins and DNA with molecular weights as high as one million Daltons can now be ionized and detected via mass spectrometry. These ionization techniques triggered the explosive development of biochemical mass spectrometry in the last decade, and two seminal contributors were recognized with the 2002 Nobel Prize.

Although both ESI and MALDI are capable of ionizing macromolecules, these methods are often complementary. Usually multiply charged ions are observed in ESI-MS, while predominantly singly charged ions are detected in MALDI-MS. ESI is very sensitive and able to handle complicated mixtures when combined with high performance liquid chromatography (HPLC), but on the other hand, MALDI is attractive due to its simplicity in sample preparation, ease of instrument operation, and high speed of data acquisition.

This dissertation describes my efforts to simplify the procedure for preparing dilute or salt-contaminated samples for MALDI-MS. The use of patterned, functional surfaces allows concentration and purification of samples directly on the modified sample probe. To place this work in context, this chapter contains a brief introduction to MALDI, including sections on the matrix, sample preparation, instrumentation, and problems that result from contamination. As my research focuses on on-probe decontamination of samples, I also present an extensive review of surface

modifications previously used for on-probe decontamination for MALDI-MS. Finally, this chapter contains a brief outline of the dissertation.

I. Introduction to MALDI

A. Roles of the Matrix in MALDI

Before the appearance of MALDI, several studies demonstrated laser desorption/ionization of nonvolatile samples.[3-5] However, the high laser intensity required to desorb macromolecules leads to strong fragmentation and fast sample consumption, thus limiting the application of this method. The addition of a matrix of small organic molecules, such as nicotinic acid[2], derivatives of cinnamic acid[6], or 2,5-dihydroxybenzoic acid[7], to macromolecular samples allows the use of much lower laser intensities to desorb nonvolatile samples. The matrix molecule to analyte ratio is very high, in the range of 10,000:1 to 100,000:1, so the analyte molecules are well separated from one another. Absorption of a pulse of laser radiation (often from an N_2 laser, wavelength 337 nm) rapidly heats the surface of the matrix crystal, leading to sudden vaporization of the top layers of the dried sample. It is under debate whether ionization of the macromolecular analytes occurs in the solid phase (primary ion formation) or during the desorption process (secondary ion formation)[8]. The presence of contaminants, such as salts or surfactants, in the sample may interfere with the co-crystalization process between the matrix and analytes by displacing analytes from matrix crystal lattices, and thus should be kept to a minimum. The matrix plays a central role in the ionization procedure, and new compounds for this purpose continue to be studied.[2,9-17]

B. Sample Preparation for MALDI-MS

For any MALDI process, the sample must be mixed with the matrix molecules. The sample preparation can be quite simple: a drop (0.5 to 1 μL) of analyte solution and a drop of matrix solution (same volume) are mixed and then deposited and dried on a stainless steel (SS) or Au sample plate (dried droplet method)[2]. In some cases, acetone is used as the solvent for the matrix solution to provide "fast evaporation" of the sample on the surface[14]. This results in small, homogeneous polycrystals on the plate. A "two-layer" method[17] can also yield uniform samples. In this case, a thin layer of seed matrix crystals is first deposited, followed by the deposition of matrix/analyte solution. These modified sample preparation methods offer better shot-to-shot signal reproducibility than the traditional dried droplet method.

C. Instrumentation (MALDI-TOF MS)

After the sample dries, the sample plate is inserted into a MALDI-time of flight (TOF) mass spectrometer (Figure 1.1). Ions formed upon laser irradiation are accelerated with a high voltage (20-25 kV) before traveling in a field-free flight tube (time-of-flight tube). Ions with the same number of charges have the same kinetic energy, but due to differences in molecular mass, they have different velocities and are thus separated by the different times they spend in the time of flight tube before hitting the detector.

D. Salt Contamination

In spite of the many successes with MALDI-MS, practical problems still exist in the application of this popular technique. One challenge is that contaminants typically found in biological extracts often degrade or eliminate analyte signals. For

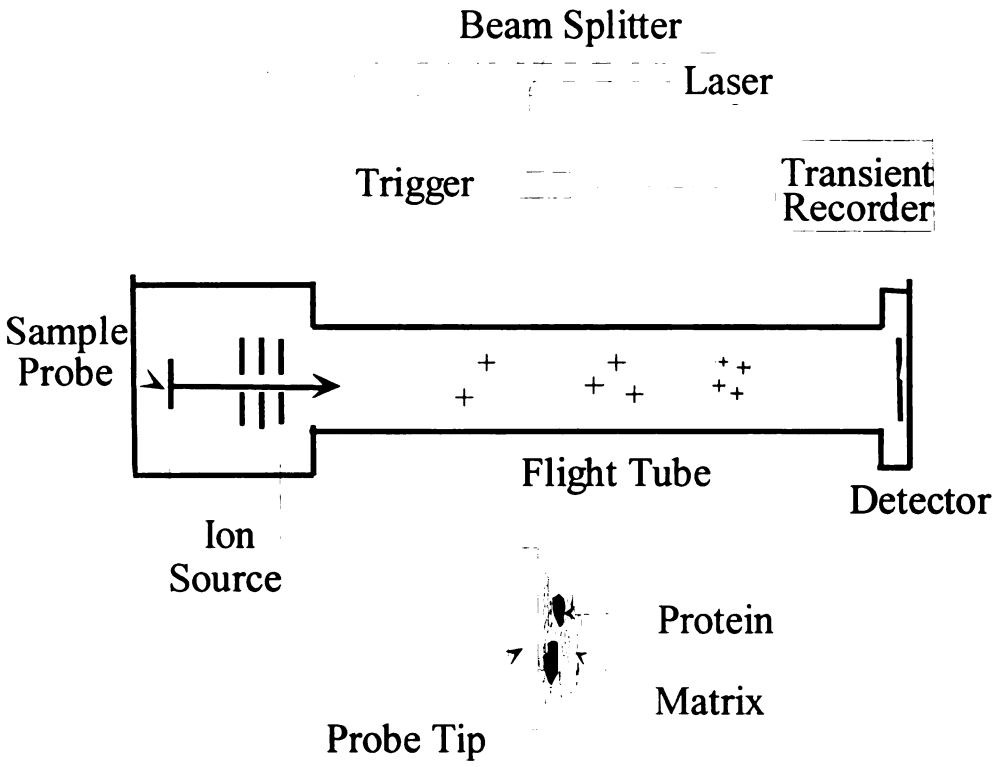

Figure 1.1 Scheme of Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometer (MALDI-TOF-MS). Adapted from *Introduction to Mass Spectrometry 3rd edition* J. Throck Watson p279

example, denaturants such as urea or guanidine-HCl are used to prevent aggregation and precipitation of hydrophobic proteins,[18] but residual amounts of these denaturants decrease or eliminate signals in MALDI-MS. The presence of stabilizing salts or surfactants also degrade the quality of mass spectra,[18] although a low concentration of these contaminants can be tolerated.[12,19,20] As reported by Kallweit et al., the quality of mass spectra usually decays when the salt concentration reaches 100 mM.[19] When the concentration of an interfering salt is around 1 M, analyte signals are usually undetectable. This is clearly shown in Figure 1.2, where a strong signal was obtained from as little as 0.5 pmol pure insulin (Figure 1.2 a); but no signal was detectable from a 5-pmol sample contaminated with 1 M sodium acetate (NaOAc), even though 10 times more insulin was present (Figure 1.2 b).

Salt contamination is also problematic in the analysis of DNA samples by MALDI-MS. As reported by Shaler et al.,[21] when salt concentrations reach 0.1 M, the peak intensity and resolution for DNA oligomers decrease due to formation of multiple cation adducts. This is shown in Figure 1.3, where the quality of the mass spectra decreases with increases in the concentration of NaCl.

To solve this salt-contamination problem, some analysts resort to HPLC to purify samples prior to MALDI-MS analysis.[22] However, HPLC is time-consuming, and purified analytes are usually present at low concentrations because of the relatively large volume of mobile phase employed for elution. Sample loss due to adsorption on the inside walls of containers during evaporation of solvent to concentrate analytes can become a serious secondary problem. Recently, micro-columns have been designed to minimize sample loss and shorten purification times.

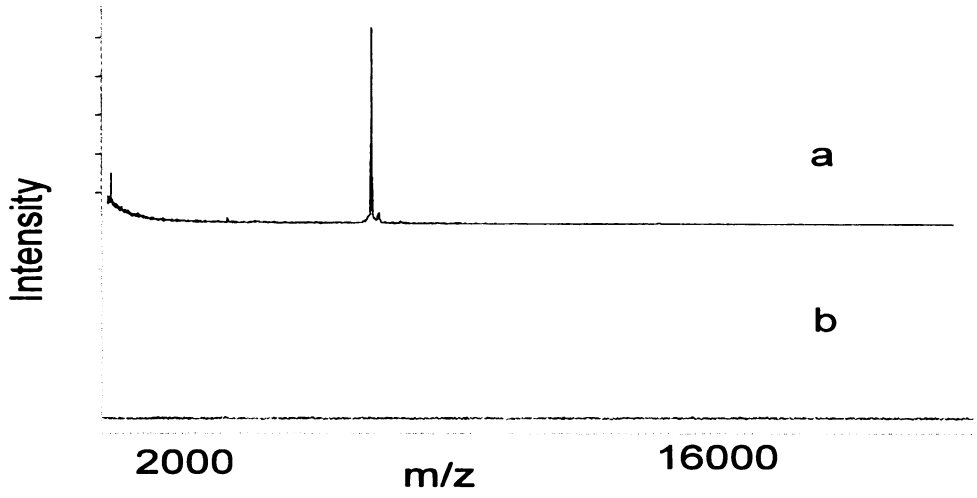

Figure 1.2 MALDI mass spectra of: (a) 0.5 pmol Insulin, no salt; (b) 5 pmol Insulin, 1 M NaOAc.

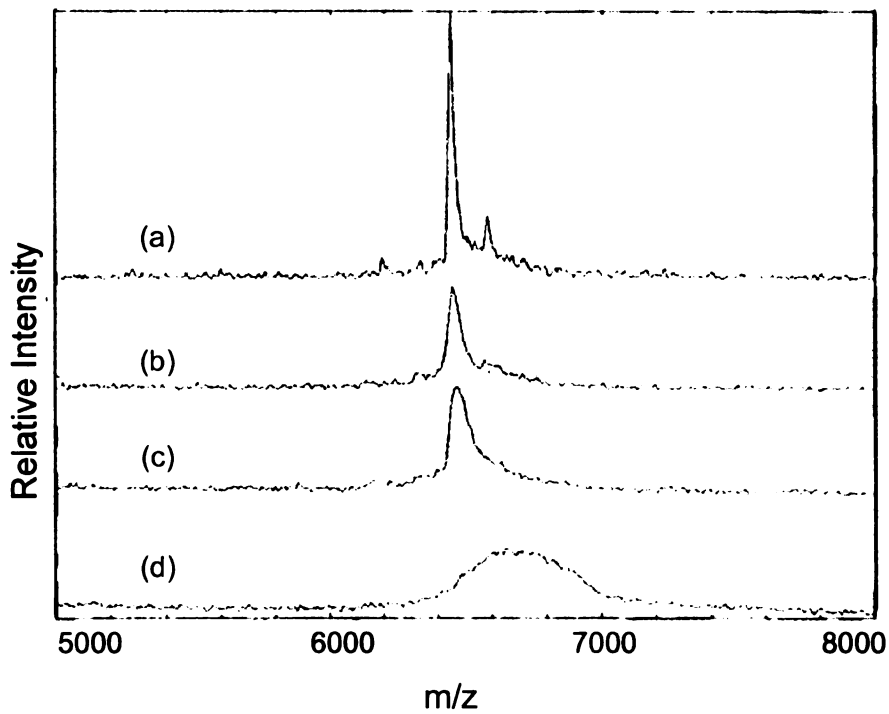

Figure 1.3 MALDI TOF mass spectra of a 21-mer of single-stranded DNA showing the effect of added NaCl: (a) control sample with no NaCl added; (b) 5 mM NaCl; (c) 25 mM NaCl; (d) 250 mM NaCl. 50 nmol of DNA 21-mer were used in each case. Adapted from T. A. Shaler et al. *Anal. Chem.* **1996**, 68, 576-579.

Commercially available Ziptip[23] (Millipore) micro-columns contain a fixed small volume (0.2 - 0.6 μL) of chromatography stationary phase at the end of 10-μL pipette tips. Analytes are concentrated on the stationary phase and eluted with a small volume of MALDI matrix solution. A similar approach using reversed-phase nano-columns was developed by Gobom et al.[24] After loading of the sample onto the nano-column and subsequent washing to remove salts, the analyte was eluted directly on to the MALDI-MS target with 50-100 nL of matrix solution. Micro-extraction chips were also used for sample clean-up and enrichment of trace peptides.[25] In comparison to conventional HPLC separations, miniaturized off-probe purification techniques have greatly simplified sample preparation. However, these micro-partitioning operations can still be time-consuming, and they add a significant cost to analyses.

II. On-Probe Sample Purification

The main focus of my research is development of new sample purification methods that are performed directly on the MALDI sample probe. This work builds on previous methods for on-probe sample purification, and this section summarizes prior work in this area. The basic strategy employed in all on-probe purification methods is to deposit sample solutions onto modified probes that allow selective adsorption of a specific class of analyte molecules. Subsequent rinsing removes contaminants such as salts, while leaving behind the analyte of interest, e.g., proteins or DNA. Finally, matrix is added to the sample prior to analysis by MALDI-MS (See Figure 1.4). This strategy eliminates the need for chromatographic separation prior to sample deposition, and should increase the sample throughput and reduce the cost of analyses.

Essentially, three types of desalting stages have been incorporated into MALDI sample platforms: films of commercial polymers, thin layers of matrix crystals, and self-assembled monolayers (SAMs)/ ultrathin polymer films. Most of these systems allow separation of contaminants from adsorbed analytes with a simple rinsing step,[12,26-33] or even without a rinsing step in some cases.[34] Mechanisms for selective binding to the sample plate range from affinity interactions to simple hydrophobic adsorption, and here we focus on methods that utilize non-specific hydrophobic and ionic interactions. Although such interactions do not allow highly selective discrimination among macromolecules, they are capable of separating contaminants such as salts and surfactants from macromolecules. This affords a simple and general method for purifying samples on the probe for analyses by MALDI-MS. Below I discuss relevant literature on decontamination with commercial polymers, thin layers of matrix crystals, and self-assembled monolayers (SAMs)/ ultrathin polymer films.

A. Decontamination Using Films of Commercial Polymers

Many types of commercially available polymer membranes have been used to selectively adsorb proteins from salty solutions as well as to interface mass spectrometry with separation techniques, such as gel electrophoresis. This section is divided into subsections according to the material employed for decontamination.

i. Polyvinylidenedifluoride and Nitrocellulose

The first type of polymer membrane utilized for protein adsorption onto a MALDI probe was polyvinylidenedifluoride (PVDF). Hefta and coworkers[35] initially deposited sample directly onto an unmodified, stainless steel MALDI probe and rinsed it with 0.1% TFA in a 30% aqueous acetonitrile solution to remove

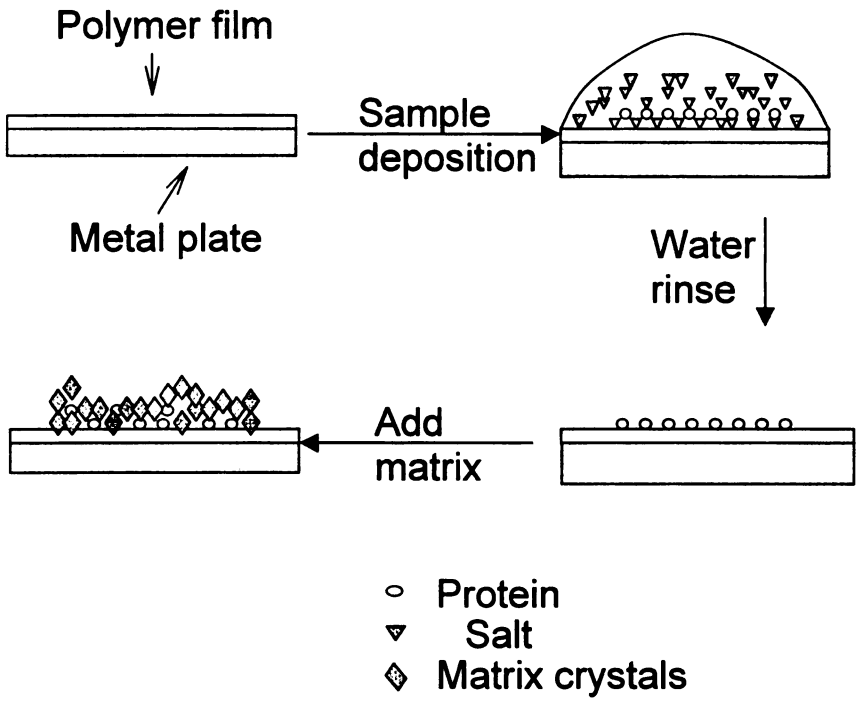

Figure 1.4 Scheme showing on-probe decontamination with a commercial membrane attached to a conventional MALDI sample plate.

contaminants. Addition of matrix allowed detection of MALDI signals for cytochrome P450, but cytochrome P450D gave no detectable signal. To improve this method, they utilized a piece of PVDF membrane as the adsorbing medium on the MALDI probe. This yielded signal for cytochrome P450D, but charging of the insulating PVDF membrane degraded the achievable mass accuracy.

To overcome the membrane charging problem, Mock et al.[12,27,36] electrosprayed a thin layer of nitrocellulose onto the probe surface, rather than using a thick piece of nitrocellulose membrane physically attached to the probe. Nitrocellulose was originally used to decontaminate samples prior to plasma desorption mass spectrometry, but in that case, a piece of nitrocellulose membrane was employed.[37] The success of desalting with electrosprayed nitrocellulose depends greatly on the experimental procedure. For example, wetting of the rough, hydrophobic nitrocellulose surface with 15% aqueous methanol facilitates good contact of the surface with the salt-contaminated sample solution during loading; drying of the sample prior to rinsing with water is also important for achieving optimal signals, especially in the case of dilute sample solutions. A high acetonitrile content (>50%) in the matrix solvent and elongated drying times were necessary to facilitate desorption of protein from nitrocellulose for incorporation into the matrix crystals.

Both Hillenkamp[38] and Fenselau[36,39] extended the utility of PVDF membranes by using them as substrates for electroblotting prior to MALDI-MS. Membranes with higher surface areas yielded spectra with higher mass resolution,[38] presumably because the more porous structure allowed incorporation of more matrix molecules to achieve an optimum matrix–to-analyte ratio. Vestling and Fenselau[40] performed on-membrane digestion of proteins, and a subsequent water rinse proved sufficient for

removal of contaminants introduced by the digestion buffer solution. Digestion of cytochrome c on the membrane yielded more detectable peptides than a similar procedure performed in solution with subsequent transfer of the digest to the MALDI sample plate. This result was ascribed to different accessibilities to cleavage sites in solution and on the membrane; however, adsorptive loss on vial walls and pipette tips during sample transfer could also be part of the problem with the solution digestion procedure.

ii. Nylon

Mass spectra from proteins adsorbed to polymer surfaces appear to depend on both the protein and the polymer composition. For example, Zaluzec et al.[32] reported only weak signals from proteins adsorbed on either nitrocellulose or PVDF; however, they showed that both ribonuclease A and trypsinogen give strong MALDI signals when adsorbed to either nylon-66 or charge-modified nylon (Zetabind). Nylon-66 also allowed analysis of a DNA-binding regulatory protein from a solution containing 6 M guanidine-HCl. (The guanidine-HCl is needed to solubilize the protein.) No signal could be observed for the analyte from the 6 M guanidine-HCl solution without purification by adsorption of the analyte on the nylon-66.

Decontamination using zetabind (Life Science Products) substrates also results in a decrease in the number of cation adducts observed in some MALDI mass spectra. When a 10-pmol sample of bovine insulin was spiked with 10-fold excess $AgNO_3$ and analyzed on a conventional MALDI plate, four Ag-ion adduct peaks were observed in the mass spectrum. Deposition of the same sample on zetabind followed by a water rinse yielded only the mono Ag-ion adduct.

iii. Nafion

Nafion (Dupont) is a perfluorinated polymer that contains sulfonate groups that serve as ion-exchange sites. Unlike the desalting procedures employed with PVDF, nitrocellulose or nylon, Nafion can bind interfering cations, and thus, in some cases, no washing step is necessary prior to adding matrix.[34,41] The ion-exchange capacity of Nafion is limited, however, and thus high concentrations of salt cannot be tolerated. Additionally, no desalting effect was observed when sample solution and matrix were premixed, possibly because the presence of an overwhelming number of protons competed with cations for binding-sites, or the surface was less effective in binding cations than protons. Nafion proved particularly effective for analysis of real biological mixtures, such as milk or egg whites, by MALDI-MS.

iv. Polyethylene (PE) and Polypropylene (PP)

Polyethylene (PE)[42,43] and polypropylene (PP)[43] provide hydrophobic surfaces for sample cleanup. Commercial membranes prepared from these polymers allowed more reproducible mass spectra from contaminated solutions than commercial PVDF and nitrocellulose membranes or C8 and C18 extraction disks. The reason for improved reproducibility with PE and PP appears to be the small uniform pores in these membranes, which allow formation of small, homogeneous matrix crystals. Usually, the residue from an aqueous solution containing as little as 0.1% SDS will eliminate MALDI-MS signals.[44] However, using a PE-modified MALDI probe as a desalting stage, Blackledge and Alexander obtained a mass spectrum of bovine serum albumin from a sample containing 0.73% SDS after vortexing the sample-coated probe in 50% aqueous methanol for 30s. (See Figure 1.5) Similarly, Woods et al.[43] observed signals from a 0.5-pmol-protein sample doped with 500 mM NaCl, 5%

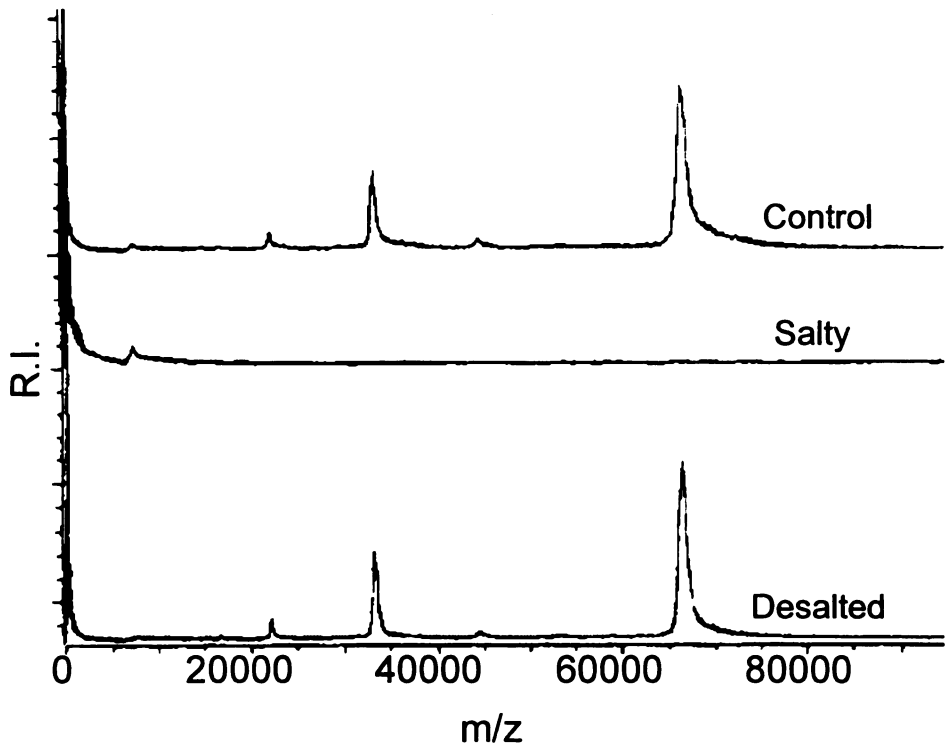

Figure 1.5 Mass spectra showing the efficacy of a PE film for adsorbing bovine serum albumin (BSA) from a solution containing excess SDS. Control spectrum (top): pure BSA applied to a PE membrane; Contaminated spectrum (middle): BSA in **0.73%** SDS applied to a PE membrane; Decontaminated spectrum (bottom): BSA in **0.73%** SDS applied to the PE membrane and subsequently vortexed in 50% methanol for **30** s. All spectra were acquired at a protein loading of 1 pmol/mm^2, and sinapinic acid was added as matrix. Adapted from Blackledge et al. *Anal. Chem.* **1995** 67, 843-848.

glycerol, and 1% Triton X-100. In the latter case, the sample was spotted onto the membrane surface, allowed to dry, and then rinsed three times with 70% aqueous methanol. No explanation was provided for the mechanism of protein binding to thepolymers, but presumably, hydrophobic interactions played an important role.

v. Polyurethane (PU)

McComb et al.[45-47] employed PU (Stevens Elastomerics) as a platform to desalt protein samples. This work was based on the study of Oleschuk and Chow[48], who showed that neutral species adsorb more strongly to PU than do charged species. Ether-type PU contains moderately polar, hard urethane domains and non-polar, soft polyether domains; proteins may interact with this material via hydrophobic interactions with the polyether domain or H-bonding with the urethane domain (See Figure 1.6). SEM images of the sample (not shown) showed that water rinsing removed NaCl crystals from the PU surface; peak shape and resolution in mass spectra of NaCl-containing, 200 pmol of myoglobin improved with increasing numbers of washes. (See Figure 1.7)

vi. Paraffin and Teflon

In the late 90s, Guo's group[34,49] utilized paraffin wax films and poly(tetrafluoroethylene) (Teflon) for sample cleanup. In the case of paraffin wax-modified probes for analysis of DNA samples, no rinsing step was incorporated in sample preparation, but salt tolerance for successful analysis by MALDI[21] increased from 5 mM to 100 mM of NaCl. The authors proposed that the hydrophobic paraffin films enhanced the rate of matrix/DNA crystallization relative to crystallization of highly polar salts.

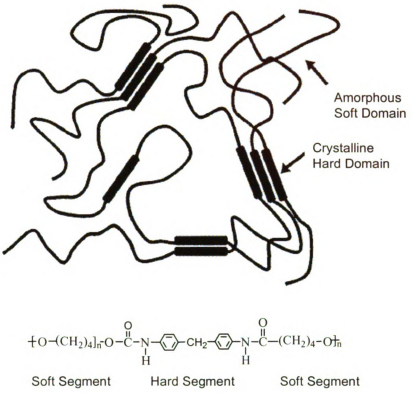

Figure 1.6 Schematic structure of a PU membrane showing the hard and soft domains. Adapted from McComb et al. Rapid Commun. Mass Spectrom. **1997** 11, 1716-1722 .

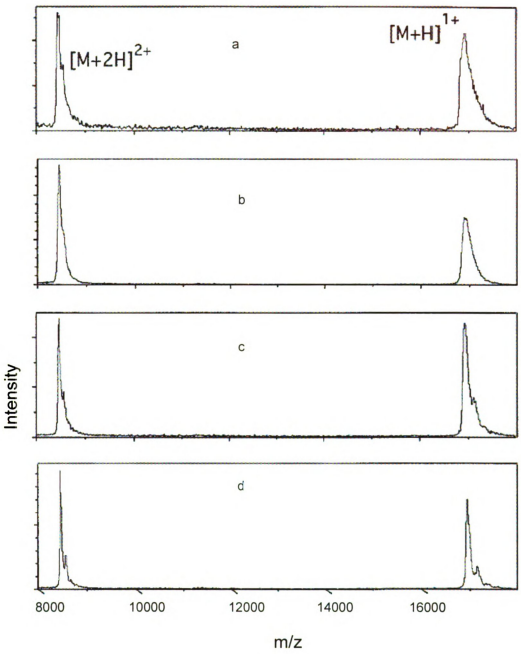

Figure 1.7 MALDI-TOF mass spectra of 200 pmol of myoglobin in the presence of 200 nmol of NaCl on a PU membrane: (a) original unwashed sample, (b) washed once with water, (c) washed twice and (d) washed three times. Figures adapted from McComb et al. Rapid Commun. Mass Spectrom. **1997** 11, 1716-1722

Guo also studied the use of Teflon as a sample loading and washing platform. A 70% aqueous methanol solution was used to rinse off salt (1 M NaOAc), and hydrophobic interactions were proposed as being responsible for retaining the protein while salts were rinsed away. Polytetrafluoroethylene (Zitex) has also been used for blotting of proteins,[50] but in that case, no purification procedure was employed.

vi. Ion-exchange Materials

Salt contamination leads to the formation of multiple cation adducts with polyanionic DNA, which results in broad peaks in mass spectra. Nordhoff et al.[51] added a few ammonium-loaded cation exchange beads to a sample droplet to replace DNA-bound sodium or potassium ions with ammonium ions, and subsequent loss of ammonia during desorption resulted in greatly simplified mass spectra. The polymer beads interfered with neither the crystallization of matrix nor the laser ablation process, and both signal intensity and mass resolution improved. Smirnov and coworkers[52] later coated MALDI probes with either polyethylenimine or polyvinylpyrollidone and used these materials to adsorb DNA from salt-containing solutions. These MALDI plates allowed both purification and concentration of DNA samples.

B. Decontamination Using Thin Layers of Matrix Crystals

In these methods, a thin layer of a relatively water-insoluble matrix, e.g., sinapinic acid, serves as the medium that selectively binds proteins in the presence of salt. Fast evaporation of solvent or crushing of raw matrix crystals produces a thin layer of micro-crystals on the MALDI probe, and exposure of the micro-crystal-coated metal

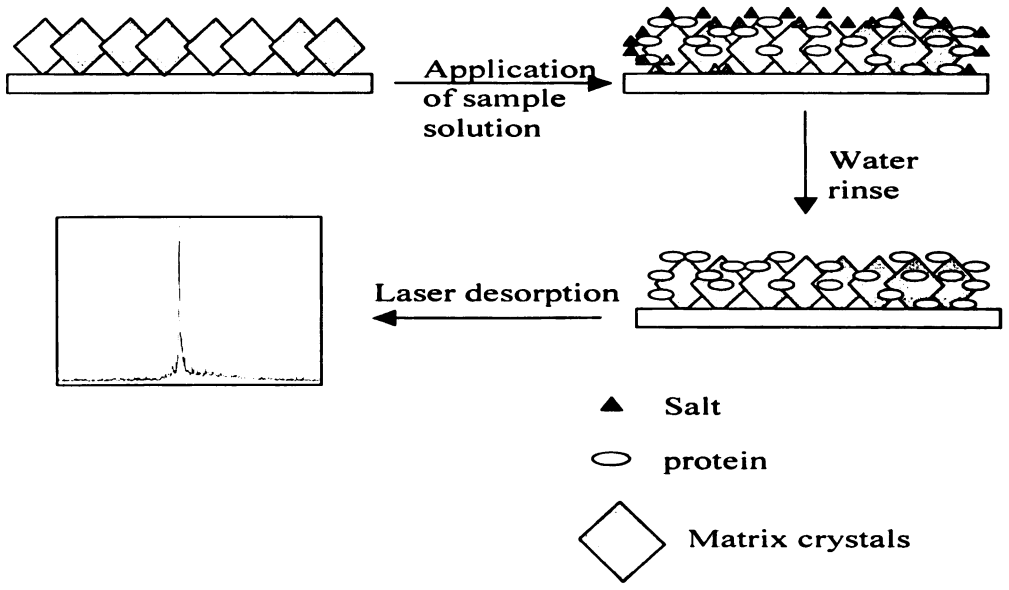

Figure 1.8 Conceptual schematic diagram showing desalting of a sample on a MALDI probe surface modified with a thin layer of matrix crystals.

surface to sample followed by rinsing with water yields a purified matrix-analyte film. (See Figure 1.8) Beavis and Chait[53] first demonstrated this concept by immersing analyte/matrix crystals into cold, distilled water to remove water-soluble contaminants. Signal intensities for the analyte did not decrease after rinsing, suggesting that the amount of protein lost from the crystals during washing was negligible. Removal of interfering salts greatly improved mass resolution, presumably because of a decrease in the concentration of cation adducts.

Below, I first review a study of the mechanism by which proteins adsorb to matrix crystals because binding to the matrix is the heart of the purification process. The subsequent section discusses the various methods for preparing MALDI probes modified with matrix crystals. Procedures employed for probe modification strongly affect the quality of mass spectra.

i. Mechanism of Adsorption to Matrix Crystals

Beavis and Bridson studied the mechanism of protein adsorption to matrix crystals using X-ray crystallography and staining of proteins with Coomassie Brilliant blue.[54] X-ray structures showed that the planar trans-sinapinic acid molecules hydrogen bonded to each other in extended sheets in the crystal lattice, while staining patterns demonstrated that proteins contacted only the crystal faces parallel to these extended sheets. The crystal plane that interacts with the protein contains no H-bond donor or acceptor atoms, suggesting that only hydrophobic interactions occur between the protein and the crystal face.

Crystallographic and staining studies also suggested a mechanism by which SDS interferes with the MALDI process. A low concentration of SDS did not affect crystallization of the matrix, but it did abolish staining of the crystal. Previous studies

showed that the hydrophobic tails of SDS molecules can bind to the hydrophobic portion of proteins to form rod-like particles[55] and change the amphiphilic nature of the protein. The hydrophobic portions of the protein would then no longer be available to bind to the hydrophobic crystal surface. The lack of binding between proteins and matrix in the presence of SDS suggests that a single layer of matrix crystals will not be effective for purifying samples contaminated with surfactants.

ii. Preparation of Desalting Matrix Crystals

1. Thin Layer of Matrix Crystals

Xiang and Beavis[11] utilized crushed crystals as "seeds" to facilitate the formation of a polycrystalline film at the base of a salt-contaminated sample droplet. In this work, they first applied a drop of matrix solution (without analyte) to the probe surface and allowed it to dry. They then crushed this compact deposit using a glass slide and brushed the surface with a tissue, leaving behind only a trace of micro-crystals. A solution containing matrix, analyte, and contaminants was then applied to the probe surface. Within several seconds, an opaque film formed at the base of the droplet, and after 1 minute, the plate was immersed in water to remove contaminants. The matrix film was stable under these rinsing conditions. This procedure yielded a strong MALDI-MS signal for 1 pmol horse skeletal muscle myoglobin even when the solution contained 6 M urea.

Cadene and Chait[56] used a modified thin-layer method to analyze membrane proteins in the presence of non-ionic detergents. They covered the MALDI probe with a small amount of matrix solution, and wiped it dry just prior to complete evaporation of the solvent to produce a thin layer of matrix crystals. Using this surface, deposition of samples containing protein, matrix, and 0.5 mM surfactant

followed by rinsing with water allowed successful analysis of several different membrane proteins by MALDI-MS. However, concentrations of surfactants higher than 0.5 mM do decrease analyte signals. Detergent tolerance is especially important in the analysis of membrane proteins because surfactants are required to prevent precipitation of these macromolecules.

Vorm et al.[57] deposited a thin layer of matrix crystals by fast evaporation of solvent to improve mass accuracy and achieve high sensitivity in MALDI-MS, presumably due to the capacity for removing salts and the formation of smaller matrix/protein crystals. They chose acetone as a matrix solvent so that the matrix/protein solution would spread quickly on the probe to produce a thin and homogeneous layer of micro crystals. The choice of matrix was limited to the less water-soluble matrices to facilitate a washing step. This method resulted in a resolving power of 5700 for medium-sized (m/z~3000) peptides. Zhang et al.[17] modified this procedure slightly by first depositing a thin film of matrix and then applying a sample droplet that contained both analyte *and* matrix. A discernible MALDI signal for 250 fmol bovine serum albumin in a sample contaminated with 1 % SDS was obtained successfully with this method.

2. Hydrophilic Spots of Matrix in Hydrophobic Polymer Layers

Using a small spot of matrix crystals in a pre-structured sample probe, Gobom et al.[58,59] improved the detection limit for analytes in salt-contaminated samples during analysis by MALDI-MS. They first coated the probe with a thin, hydrophobic polymer layer that contained an array of tiny holes (400 μm in diameter). Growth of a thin layer of matrix crystals only in the holes provided localized adsorption sites for the protein or peptides, while keeping the surrounding region hydrophobic. Because

of the difference in hydrophobicity between the matrix crystals and the surrounding hydrophobic polymer region, the sample droplet evaporated to the size defined by the localized spot of crystals, concentrating the analyte molecules. As with other methods, a rinsing procedure washes away contaminants and leaves protein behind. The small spot sizes resulted in 10-20 fmol detection limits, even for the analyte in salt-contaminated samples.

C. Decontamination using SAMs and Ultrathin Polymer Films

The use of SAMs and ultrathin polymer films for modifying MALDI probes allows tailoring of this interface for purification of protein samples. Additionally, the minimal thickness of these coatings should alleviate problems with charging of the modified probes. Several studies demonstrated the attachment of a monolayer of antibodies to a MALDI probe to specifically bind an antigen in the presence of contaminants.[60-62] Those studies fall into the category of sample cleanup with affinity interactions, and thus are not in the scope of this project. Here I discuss the fabrication of monolayer/ultrathin polymer films that can adsorb proteins from contaminated solutions via hydrophobic or electrostatic interactions.

i. Decontamination using Hydrophobic Interactions

SAMs of alkanethiols provide a well-characterized surface[63] for implementing hydrophobic interactions in the purification of analytes from sample solutions prior to analysis by MALDI-MS. Brockman et al.[64] modified MALDI probes with a monolayer of octadecyl mercaptan (OM) by immersing a clean gold MALDI probe into an ethanolic OM solution. (See Figure 1.9)

Analysis of samples using these probes involved the standard procedure described above: deposition of sample, rinsing, deposition of matrix solution, evaporation of

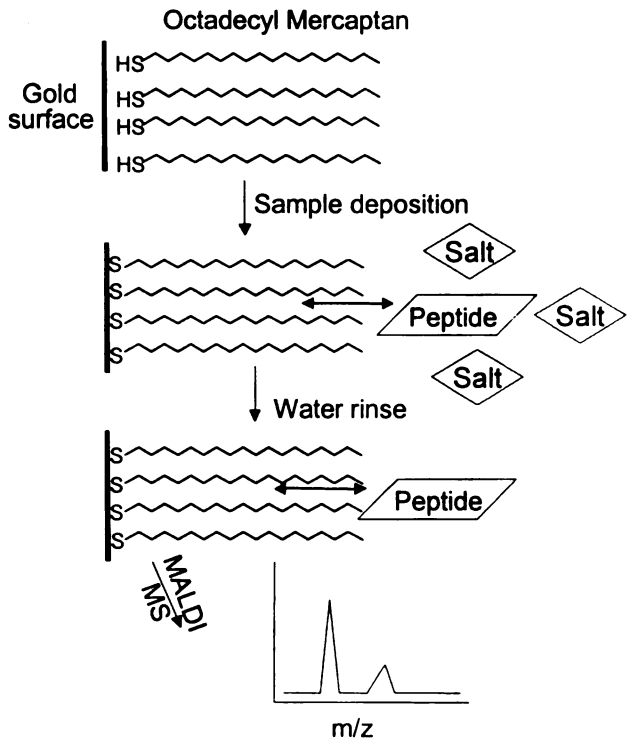

Figure 1.9 Conceptual illustration of SAM formation and subsequent mechanism of protein adsorption.Adapted from Brockman et al. *Anal. Chem.* **1997** 69, 4716-4720.

solvents, and analysis of the crystalline residue by MALDI-MS. Using this procedure, analyte signals are rather difficult to obtain, and the analyst must search all over the modified sample probe for a few spots that yield reasonable single-shot spectra. Brockman et al. speculate that this occurs because a limited contact area between the sample droplet and the probe surface (high contact angle) results in limited adsorption of the analyte. Improved reproducibility results from immersing the SAM-modified probe into the salt-containing protein solution overnight (8 h). However, the longer immersion time greatly decreases sample throughput, and additional analyte solution is necessary to cover the probe. A subsequent study showed that the amount of protein binding to the SAM is independent of analyte concentration in solution, but dependent upon immersion time.[65] This happens presumably because the SAM has a limited binding capacity and, thus, increasing the protein concentration may not increase the amount of protein adsorbed to the surface. However, after the formation of the first protein layer, some loose layers of protein may form by attaching themselves to underlying proteins via H-bonding, electrostatic interactions, or hydrophobic interactions. The formation of these "loose" layers may be time-dependent, and thus, longer immersion times lead to higher adsorption levels.

ii. Decontamination Using Electrostatic Interactions

Because of the slow protein adsorption on hydrophobic monolayers, Orlando and coworkers began investigating the utility of ionic monolayers for extracting proteins and peptides from salty solutions prior to analysis by MALDI-MS.[29] Remarkably, ionic monolayers successfully extracted proteins from solutions contaminated with 20% Triton X-100, 8 M urea, and saturated sodium acetate. (See Figure 1.10)

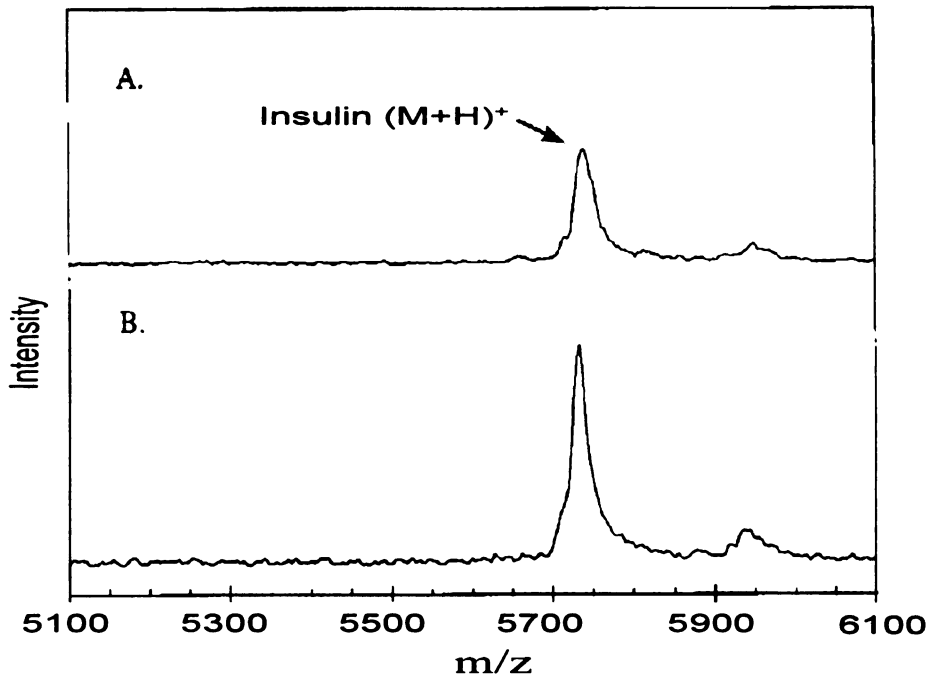

Figure 1.10 Ion-pairing solid phase extraction MALDI/MS spectrum of insulin (2 pmol/mL) from a solution of the peptide saturated with sodium acetate. These spectra were acquired using (A) a strong cation-pairing surface (3-mercapto-1-propanesulfonic acid) and (B) a strong anion pairing surface (2-aminoethanethiol hydrochloride). Figure was adapted from Warren et al. *Anal. Chem.* **1998** 70, 3757-3761.

Results from these two experiments suggest that the mechanism for protein extraction by these monolayers is ion pairing and not hydrophobic interactions or other forces. First, washing the dried salty sample with organic solvent rather than deionized water has no significant effect upon analyte signal intensities. If hydrophobic interactions dominated protein extraction, rinsing with organic solvent should eliminate, or at least decrease, protein or peptide signals. Second, the pH of rinsing solutions affects the amount of protein bound to monolayers containing weak acid groups. Rinsing with pH 2.3 solutions removes peptides bound to carboxylate-terminated surfaces, but has little effect on peptide adsorption at sulfonate-terminated surfaces. At pH 2.3, carboxylate groups should be protonated (neutral), while strongly acidic sulfonate groups will be deprotonated (negatively charged). Thus, results from this experiment suggest that ion pairing is the main driving force for peptide adsorption. Cations in contaminated solutions may compete with proteins/peptides for adsorption sites, but the peptides/proteins have multiple binding sites, and hence, achieve a stronger overall interaction with the charged surface.

Figure 1.10 shows that both positively (mercaptoethylamine) and negatively (mercaptopropanesulfonic acid) charged surfaces are capable of extracting insulin from highly contaminated solutions. This result raises the question as to how electrostatic interactions can occur between a single protein and both positively and negatively charged monolayers. Additionally, insulin contains only two positively charged residues and would not be expected to bind strongly to a sulfonated surface in the presence of a large excess of cations. These conditions suggest that binding of proteins to charged monolayers is not driven solely by electrostatics; entropy changes due to release of bound water upon protein binding may also favor adsorption.

One of the main problems in using SAMs for decontamination of samples prior to analysis by MALDI-MS is their limited binding capacity, which results in weak signal intensities for the analyte. To address this problem, Zhang and Orlando immobilized polylysine chains onto a gold surface via a succinimide-containing monolayer.[33] Signal intensities in MALDI mass spectra of proteins adsorbed to this surface increased with the molecular weight of the polylysine. This result probably occurs because an increase in polylysine molar mass translates into an increase in immobilized amine groups on the probe, thereby providing more binding sites for protein adsorption.

III. Dissertation Outline:

Above I reviewed the wide variety of on-probe decontamination methods used prior to analysis by MALDI-MS. Below I will describe the approach I employed to achieve both purification and concentration of proteins (chapter two), peptide mixtures (chapter three), and DNA (chapter three) on the MALDI probe. Chapter 4 will present my most recent work that aims at enhancing the detectability of phosphopeptides via selective, on-probe adsorption.

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Chapter Two: Patterned Monolayer/Polymer Modified Metal Surfaces as Sample Platforms for Analysis of Dilute or Salt-Contaminated Protein Samples by MALDI-MS

I. Introduction

This project was partially inspired by Nordhoff and coworkers' research on prestructured MALDI sample supports,[1] where 200-μm diameter gold spots on hydrophobic Teflon provide anchors for dilute samples during analyses by MALDI-MS. Due to the water-repellant nature of hydrophobic Teflon, the sample droplet stays preferentially on the hydrophilic gold. With the evaporation of solvent, analytes are concentrated in the small area defined by the 200-μm gold spot. Compared to the use of a conventional metal MALDI plate (stainless steel or gold), where the sample droplet spreads out in the 2-mm diameter sample well (as illustrated in Figure 2.1), use of the prestructured sample support can decrease the detection limit for an analyte by more than an order of magnitude.

However, only pure samples can be analyzed using this modified probe, as the contaminants would be concentrated with the analyte and would cause an even more serious problem during the desorption/ionization process. I report here the modification of a conventional MALDI plate with a patterned self-assembled monolayer (SAM) of hexadecanethiol (HDT) prepared by micro-contact printing.[2] In one manifestation of these patterned probes, small (200-μm diameter) spots of hydrophilic bare gold patterned in the hydrophobic HDT SAM provide anchors for droplets of the sample solution and allow subsequent concentration of samples during solvent evaporation (in a similar fashion to use of a prestructured sample support). This process yields both decreased detection limits and increased signal reproducibility, as seen in other work with use of small sample spots,[1,3,4]

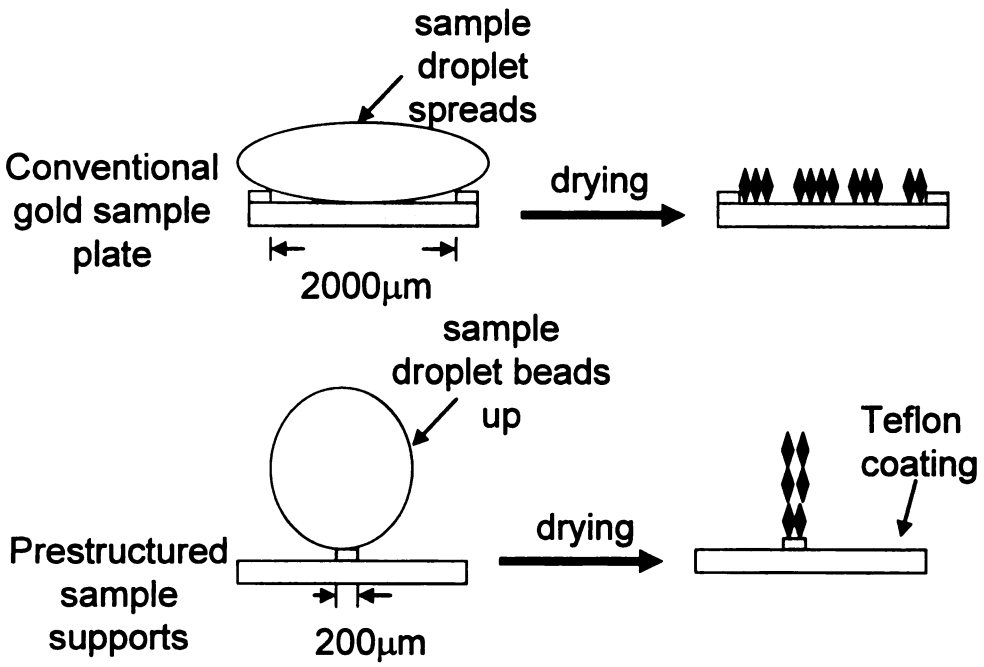

Figure 2.1 Comparison of sample deposition on conventional (top) and prestructured (bottom) sample supports.

but these patterned substrates can be conveniently prepared in nearly any laboratory.

A second, more versatile manifestation of the patterned gold surface allows protein purification to be combined with concentration of the solute to a small spot. This method achieves low detection limits even with contaminated samples. In this manifestation, using a method developed by Crooks and coworkers, I graft hydrophilic poly(acrylic acid) films to the bare gold spots in the otherwise hydrophobic surface presented by the SAM of hexadecanethiol.[5,6] Although specially modified sample plates with patterned hydrophilic spots are already commercially available (Anchorchip, Bruker, *http://www.daltonics.bruker.com/applications/shared/maldiapp1070anchorWEB.pdf*), the use of PAA allows decontamination of protein samples along with lower detection limits and increased reproducibility. Thus, the PAA-patterned sample supports combine the advantages of patterned substrates[1] and sample decontamination.[7] Additionally, PAA provides a versatile support surface because it can be derivatized with a wide variety of functional groups, as will be seen in chapter 3.[5]

I also report reflectance FTIR spectroscopy studies of the mechanism of protein binding to PAA films. Infrared spectroscopy provides a powerful tool for measuring the amount of adsorbed protein in the film as a function of sample solution conditions such as pH. These studies show that protein binding to PAA

occurs primarily due to electrostatic interactions, and thus is dependent upon pH and the concentrations of proteins and salts. Additionally, IR spectra show that the acidic matrix solution removes proteins from PAA films, allowing co-crystallization with the matrix. These studies also demonstrate that IR spectra of protein adsorbed on the polymer-modified surface correlate well with MALDI-MS signal intensities obtained from corresponding HDT SAM/PAA surfaces after addition of matrix.

II. Experimental Section

A. Materials and Solutions

Dilute (200 amol/μL to 18 pmol/μL) protein solutions were prepared in deionized H_2O (milli-Q, 18 MΩcm), 8 M NaOAc, or 1 M NaOAc within 1 h of the corresponding experiment. For solutions in deionized water, pH values were adjusted by addition of 1 M HCl or 1M NaOH. All proteins and chemicals were obtained from Sigma or Aldrich unless noted otherwise. Gold-coated substrates (200 nm of gold sputtered on 20 nm of Cr on Si(100) wafers) were prepared by Lance Goddard Associates (Foster City, CA).

B. Fabrication of Patterned Substrates

Poly(dimethylsiloxane) (PDMS) stamps were prepared according to the methods developed by Whitesides and coworkers.[2] Briefly, the desired pattern

(200-μm spots separated by 5 mm) was printed on an ordinary transparency sheet using a laser printer. A "pre-baked" (30 min, 60 °C) photoresist (AZ P4210, Clariant) spin-coated on a Si wafer was then selectively exposed to UV light (Hg lamp) using the transparency as a mask, and the photoresist was immersed in developer (AZ 421K, Clariant) for 1 min to yield 200-μm diameter protrusions. After "post-baking" (30 min, 60 °C), a PDMS elastomer solution (Sylgard 184, Dow Corning) was poured onto the photoresist template and cured overnight at 60°C. The PDMS stamp, consisting of 200-μm diameter indentations, was then peeled off the photoresist. The stamp can be used more than 500 times without losing the patterning resolution.

Prior to the printing of patterns, gold-coated substrates were first cleaned for 15 min in a UV/ozone cleaner (Boekel 135500), rinsed with deionized water for ~10 s, and dried with N_2. PDMS stamps were "inked" with HDT by swabbing the stamp with a ~10 mM solution of HDT in ethanol, and then dried by a stream of N_2. The stamp, still moistened with residual HDT, was then gently contacted to the gold substrate (as suggested early in Figure 2.2), and air bubbles were removed by lightly pressing on the stamp. After ~30 s, the stamp was removed, leaving a thin layer of HDT. Excess HDT was rinsed away with ethanol and water to yield a pattern of gold spots in the HDT SAM (HDT SAM/Au).

To further modify the patterned HDT SAM surface (step 2 in Figure 2.2), we chemically grafted PAA onto the bare gold spots according to the procedure of

Crooks and coworkers.[6] Briefly, we dipped the gold-coated wafer into 1 mM mercaptoundecanoic acid (MUA) in ethanol for 60 s, and then rinsed it with water and ethanol. After activating the MUA using ethyl chloroformate, we chemically attached amino-terminated poly(*tert*-butyl acrylate) to the MUA linker. Finally, we hydrolyzed the *tert*-butyl ester groups using methanesulfonic acid in dichloromethane.

C. Sample Preparation Prior to Analysis by MALDI-TOF-MS

For analysis of dilute, pure protein samples on HDT SAM/Au-patterned surfaces, 0.25-0.5 μL of the sample solution was applied to a bare gold spot, after which an equal volume of matrix solution (10 mM 2,5-dihydroxybenzoic acid (DHB) in deionized water) was added and the mixture was allowed to dry. When using HDT SAM/PAA surfaces, 0.25 μL of salty protein solution was deposited on the PAA spot and the droplet was allowed to stand for 3-5 min. Before the drop dried, the surface was rinsed with copious amounts (~10 mL) of MilliQ water and dried with N_2. Subsequently, 0.25 μL of matrix solution was added and allowed to dry. The gold wafers were attached to a disposable MALDI plate using double-sided tape or superglue. When the sample preparation process required rinsing with water, the wafers were always attached to the plate

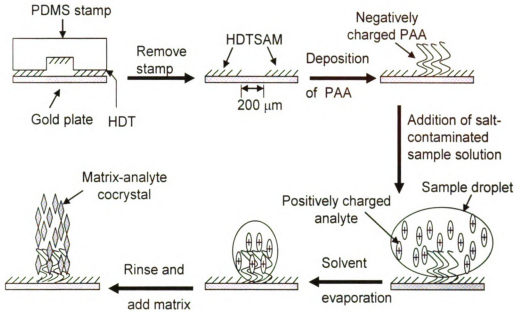

Figure 2.2 Schematic flowchart for the preparation of a patterned HDT-SAM/PAA sample probe and subsequent on-probe cleaning and concentrating of a salt-contaminated protein sample for analysis by MALDI-MS.

after drying with N_2, as otherwise it took a long time (~30 min in extreme cases) to evacuate the sample inlet system prior to analysis by MALDI-MS.

D. Instrumentation

Mass spectra were obtained with a PE Biosystems Voyager Elite or STR MALDI TOF mass spectrometer using an accelerating voltage of 25 kV, a 95% grid voltage, 0.05 % guidewire voltage, and an extraction delay time of 100-150 nsec to accumulate ion current associated with 30-50 laser pulses. Ellipsometric experiments were performed using a rotating analyzer spectroscopic ellipsometer (J.A. Woollam) and assuming a film refractive index of 1.5. Reflectance FTIR spectra were obtained with a Nicolet Magna 560 spectrophotometer with a Pike grazing angle (80°) accessory. The spectrophotometer was housed in a glove box to minimize interference from water vapor.

III. Results and Discussion

A. Patterned HDT SAM/Au surfaces as sample plates

The hydrophilic bare gold spots in HDT SAMs provide anchors for the sample/matrix droplet, allowing concentration of a sample by deposition of the solute to a smaller surface area during the evaporation of solvent. This improves both the detection limit and signal reproducibility in MALDI-MS. The detection limit (signal-to-background ratio $\geq$ 3) for ribonuclease A (RNase A) in

conventional MALDI (2-mm diameter sample wells) is approximately 100 fmol, while the detection limit using a patterned HDT SAM/Au surface is about 1 fmol as illustrated by the mass spectra in Figure 2.3.

Detection limits also decrease for insulin, insulin chain A, and insulin chain B (0.3-3 fmol, see Figure 2.4, for representative spectra). From the spectra in Figure 2.4, one may have the impression that the detection limit can be further lowered as the signal-to-noise ratio is much higher than 3:1. Indeed, signals from 0.2 fmol insulin and other small peptides have been observed, but not on a routine basis; this may be due to the fact that at very low concentrations, most of the peptides are adsorbed to the pipette tip and, thus, are not available for analysis in MALDI-MS. A similar phenomenon was observed in another study of a prestructured sample probe.[1] In this chapter, I provide only the spectra that can be obtained routinely. The surface area in a 2-mm sample well is 100 times that of a patterned 0.2-mm diameter gold spot, so in principle, the sample concentration on the patterned surface should be 100 times higher than that on a conventional MALDI plate when equal amounts of analytes are deposited. This surface area ratio may explain the improvement in detection limit for RNase A when using the patterned HDT SAM/Au surface. However, detection limits for smaller proteins decrease by a factor of only ~10 when using the patterned plate, indicating that surface area is not the only variable affecting sensitivity. Previous studies with small sample spots also showed a ~10-fold decrease in detection

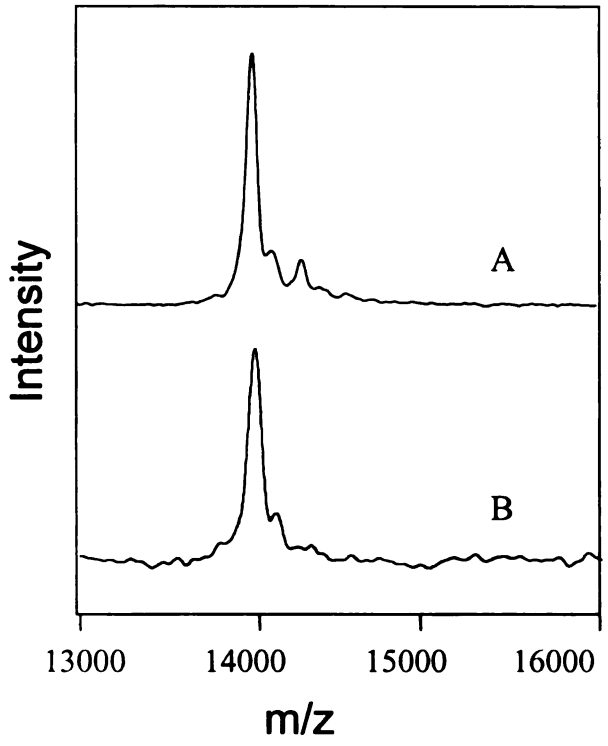

Figure 2.3 MALDI mass spectra of RNase A obtained using different sample probes. (A) 600 fmol RNase A using a conventional 2-mm diameter sample well (70 mM DHB as matrix); (B) 1 fmol RNase A using a 200-mm diameter Au spot in a patterned HDT SAM (10 mM DHB as matrix).

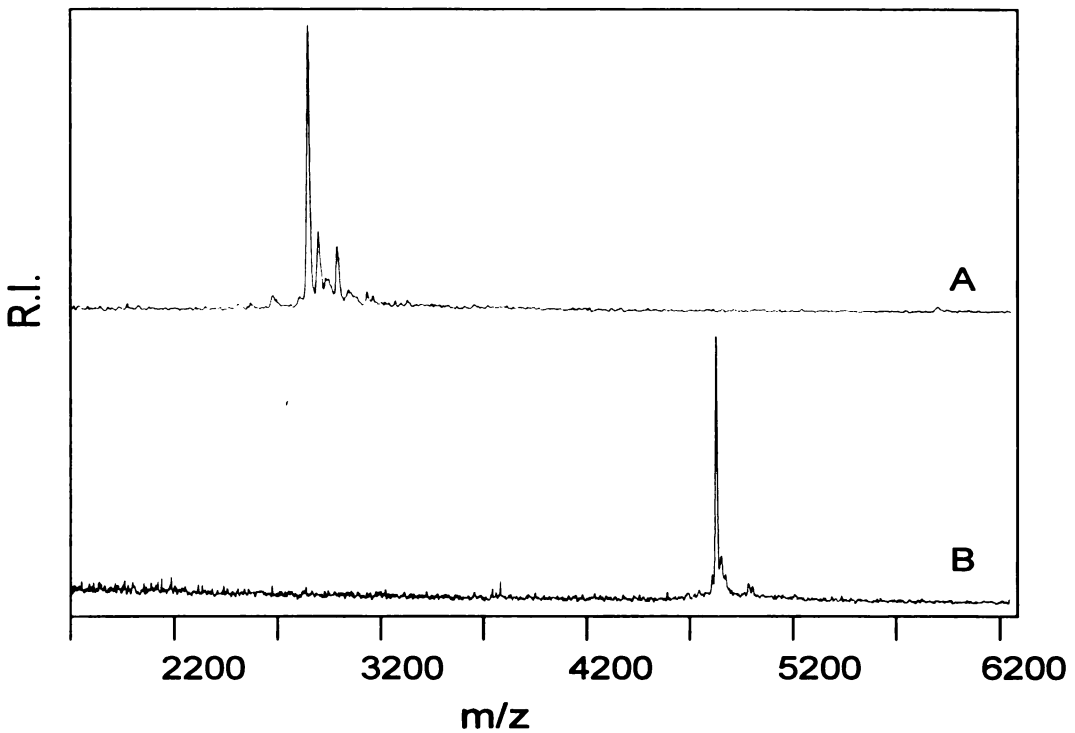

Figure 2.4 Mass spectra of (A) 3 fmol insulin chain B and (B) 2.5 fmol insulin. Spectra were obtained from 200-mm diameter Au spots in HDT SAMs (10 mM DHB as matrix).

limits.[1]

Another important experimental factor is the matrix solution because both matrix and analyte molecules are concentrated when solvent evaporates. If the initial matrix concentration is too high, the size of the solid residue is usually larger than the dimensions of the underlying gold spot, and it is difficult to obtain reproducible signals. The matrix solvent is also an issue. If the solvent evaporates too quickly, as can happen with acetone for example, the size of the solid residue is often greater than the size of the bare gold spot. Usually, matrix solutions made with water, 70% acetonitrile and 30% water, or 50% acetone and 50% water give reasonable sample drying rates and small sizes of solid residues.

As the sample spot size approaches the area illuminated by the laser, signal reproducibility also improves compared to that achieved from a conventional MALDI sample plate. For replicate measurements with RNase A, insulin, insulin chain A, and chain B made at several different Au spots on a patterned probe, the relative standard deviation in signal intensity is less than 30% (four samples analyzed in each case, see Table 2.1). In these experiments, the operator ensured that the laser beam illuminated matrix crystals, and signal was observed in every case. As the sample size is about 3 or 4 times larger than the cross section of the laser beam (i.e., 25-33% of the sample is illuminated on each laser shot), usually 3 or 4 spectra (a spectrum consists of the averaged signal following 30 to 50 laser shots) can be obtained within one sample well, and we can get usable signal in

Table 2.1 MALDI-MS signal intensities for replicate samples on patterned HDT SAM/Au surface

Protein	Intensity	Intensity	Intensity	Intensity	Standard deviation	Average	Relative Standard Deviation
Insulin chain A (16 fmol)	3720	3811	4764	4769	579	4266	14
Insulin chain B (16 fmol)	23000	21000	16000	21000	2986	20250	15
Insulin (32 fmol)	27000	15000	16000	22000	5598	20000	28
RNase A (50 fmol)	2206	4067	3564	2789	823	3156.5	26

most cases (>75%). Excluding spots where no measurable signal is obtained (<25%), the relative standard deviation of measurements at different spots within the same 200-μm diameter sample well is less than 30%. We have not been able to obtain similar reproducibility with the same dried-droplet-method sample preparation procedure on a stainless steel probes. In the latter cases, variation in signal intensities can be as high as several orders of magnitude; very often no signals can be detected in specific sample locations (>95% in worst cases, i.e., signal might be detected from as few as 1 position out of 20). The variation in signal intensities from the patterned substrates is comparable to that obtained previously using probes modified with 300-μm diameter matrix/nitrocellulose spots, in which case samples were deposited with a piezoelectric pipette.[3]

The ideal diameter of the solid residue should be even smaller than 200 μm so that the laser beam would illuminate the whole sample area. (The laser beam that we use has a diameter of approximately 100 μm, and the intensity of the laser is less at the edge of the beam than in the center.) Unfortunately, the sample deposition process provides an effective minimum limit on the size of the hydrophilic gold spot. To detach the sample from the pipette tip, there must be a sufficiently strong attraction between the surface and the droplet, and this attraction is too weak to deposit samples on hydrophilic gold spots with diameters <100 μm. A similar phenomenon has been reported in the use of prestructured supports.[7] We have experimented briefly with 500-, 200-, and 100-μm Au spot diameters, and the 200-μm diameter Au spots yielded the most reproducible signals and lowest detection limits.

B. Patterned HDT SAM/PAA Surfaces as Sample Plates

Although patterned HDT SAM/Au substrates give low detection limits, they are not capable of decontaminating protein solutions. In fact, contaminants will be concentrated with this system. To prepare patterned surfaces capable of both concentrating and desalting solutions, we grew PAA films in the bare Au holes of patterned HDT SAMs (see Figure 2.2). In the decontamination procedure, we deposited ~0.25 μL of a salt-containing solution on the PAA spot, waited for 3-5 minutes to allow partial evaporation of solvent, rinsed the spot with water, dried it

with N_2, and subsequently added matrix solution to the sample residue. The sample size, after evaporation of matrix solvent, is about the size of the underlying PAA spot. Using these films, the detection limit for 1 M NaOAc-contaminated insulin is approximately 25 fmol (Figure 2.5); this value is 20- to 100-fold lower than detection limits reported with the use of non-patterned polylysine surfaces[8] or SAM surfaces.[9]

Patterned HDT SAM/PAA sample plates also allow low (20-50 fmol) detection limits for insulin chain A, insulin chain B, and RNase A in the presence of 1 M NaOAc. Both conventional MALDI sample wells and patterned HDT SAM/Au plates (with or without rinsing) give little or no signal with application of as much as 1 pmol of RNase A in the presence of 1 M NaOAc. Detection limits obtained using patterned HDT SAM/PAA sample plates for salty solutions are even lower than uncontaminated-solution detection limits obtained using conventional MALDI plates. This is due, again, to concentration of the protein on the small sample spot. Although contaminants in the sample solution are also concentrated during evaporation of the solvent, they can be rinsed off easily with water while the analyte molecules remain bound to PAA.

C. Mechanism of Desalting and Incorporation of Proteins into the MALDI Matrix

In the decontamination and concentration process, positively charged

proteins most likely bind to the surface due to electrostatic interactions with PAA, which contains many carboxylate groups at pH values above its pKa (≅4.5).[10] Because many proteins can contain multiple positive charges, they adsorb to the negatively charged surface more strongly than simple salts, and thus salts can be removed preferentially by rinsing with water. Subsequent addition of an acidic MALDI matrix should remove the protein from the film by protonating PAA, thereby eliminating electrostatic interactions. Reflectance FTIR spectroscopy studies confirm this mechanism of protein binding and incorporation of analyte into the MALDI matrix.

If electrostatic interactions are essential for decontamination, protein absorbances in IR spectra and signal intensities in mass spectra should depend on the pH of the sample solution. At pH values where the protein is highly positively charged and the film is negatively charged, maximum adsorption should occur. Thus, the solution pH should be below the pI of the protein (9.7 for RNase A, calculated by GPMAW software), but above the pKa of PAA (4.5). Figures 2.6 and 2.7 show that maximum amide absorbances occur at an adsorption pH of ~5.5. (Amide absorbances should be approximately proportional to the amount of adsorbed protein.) The absorbance maximum at pH 5.5 represents a compromise between achieving the highest number of positive charges on the protein and the highest negative charge density in the film. These IR absorbance data are consistent with mass spectral data, which also show maximum signal intensity

when the protein solution has a pH of 5.5 (Figure 2.7). The pH for maximum adsorption will, of course, vary somewhat from protein to protein, depending on pI values.

Protein adsorption should also depend on the concentration of both protein and salt in sample solutions. Figure 2.8 shows a plot of amide absorbance as a function of the concentration of RNase A in the solution to which the sample plate was exposed (solution pH of 7.0). The plot suggests that adsorption increases linearly as RNase A concentrations increase from 0.1 to 10 pmol/μL. Compared with the acid carbonyl absorbance (~0.003 for a protonated PAA film), the amide absorbance (0.05) for a sample plate exposed to 20 pmol/μL RNase A solution suggests that multilayers of protein are forming in highly extended PAA chains. Ellipsometric measurements confirm multilayer formation as film thickness increases 10-fold (from 25 Å to 250 Å) after exposure

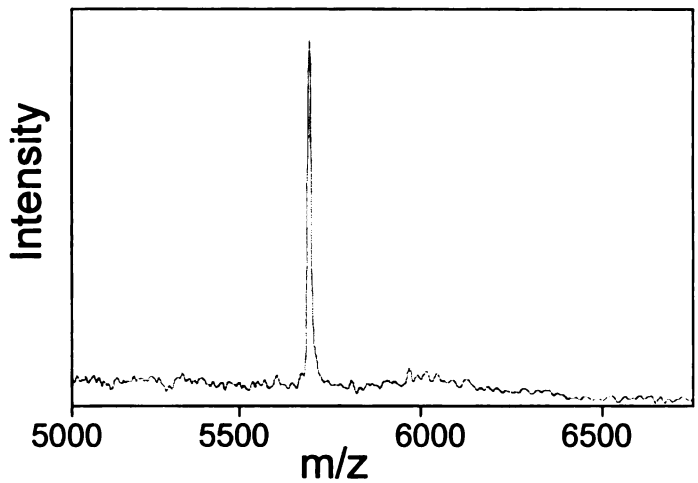

Figure 2.5 MALDI mass spectrum of insulin adsorbed from 0.25 μL of solution containing 25 fmol insulin and 1 M NaOAc. The sample was applied to a 200-μm diameter PAA spot in a HDT SAM and rinsed with water. DHB (10 mM) was added as matrix.

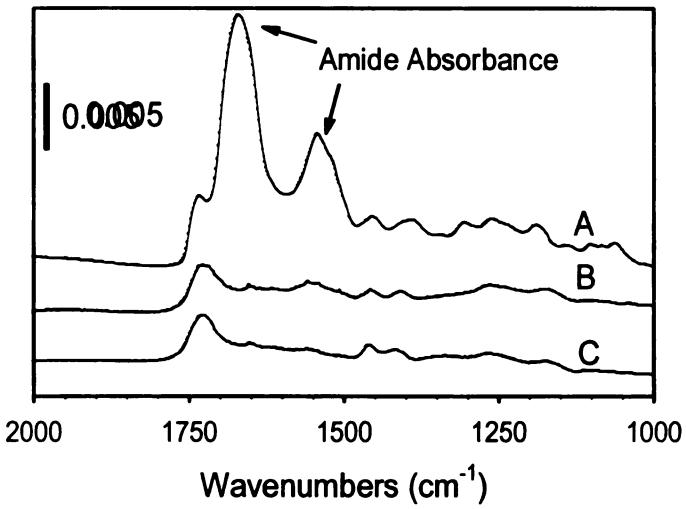

Figure 2.6 Reflectance FTIR spectra of PAA films after exposure to a 1-μM RNase A solution for 30 min at different pH values: (A) pH 5.5; (B) pH 1.6; (C) pH 12.2. Films were rinsed with water prior to measurements. In these studies, substrates were not patterned, but completely coated with PAA to provide enough capacity to achieve a sufficient signal-to-noise ratio in the IR spectrum.

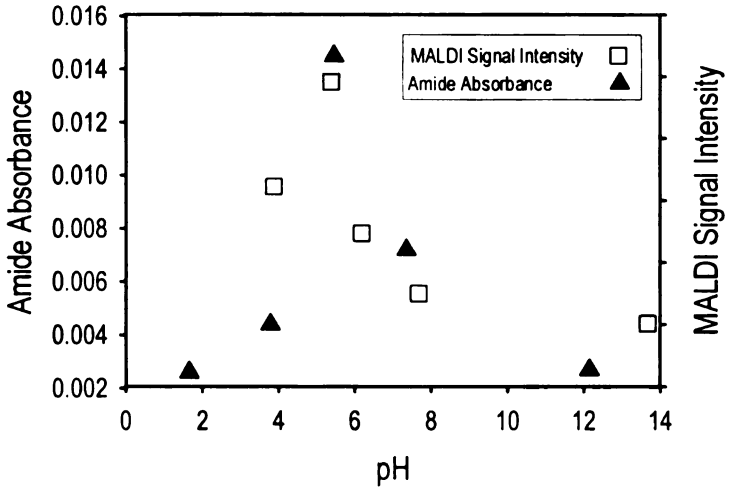

Figure 2.7 Amide absorbances in reflectance FTIR spectra and the protonated protein signals obtained using MALDI-MS after exposure of sample plates to RNase A solutions (1 μM, no salt) at different pH values, followed by water rinsing. Mass spectra were taken on patterned HDT SAM/PAA plates (10 mM DHB as matrix) and FTIR spectra were measured on plates coated only with PAA.

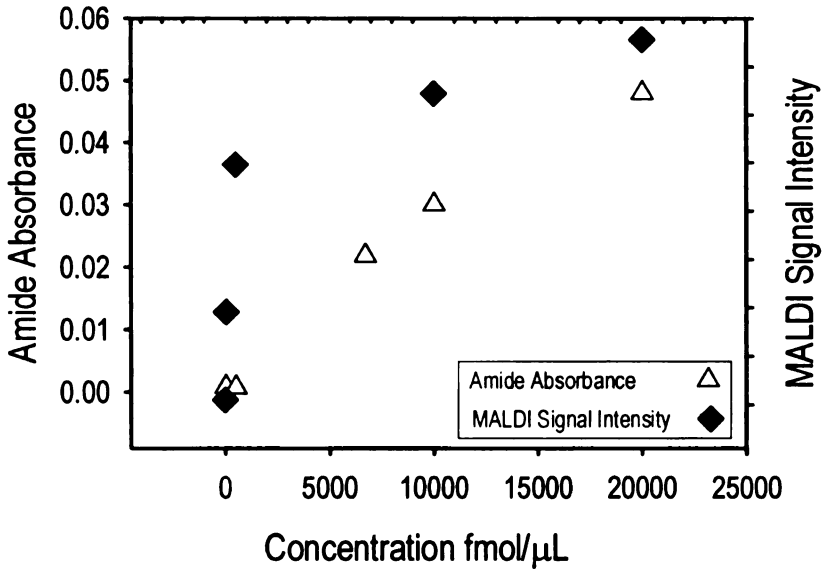

Figure 2.8 Amide absorbances in reflectance FTIR spectra and the protonated protein signals obtained using MALDI-MS after exposure of sample plates to RNase A solutions at different concentrations (pH 7, no salt), and water rinsing. Mass spectra were taken on patterned HDT SAM/PAA plates (10 mM DHB as matrix) and FTIR spectra were measured on plates coated only with PAA.

of PAA to 20 pmol/μL of protein. Evidence for the formation of multilayers helps to explain why patterned surfaces are so successful in concentrating protein analytes from salty samples.

MALDI signals should be proportional to the amount of protein adsorbed on the surface. However, MALDI signal intensities increased more rapidly with concentration than did amide absorbances. This may be due to the fact that in the MALDI experiments, the solution is concentrated on the small spot, while in the IR experiments, no concentrating of protein occurs (the non-patterned PAA-coated sample plate was immersed in a protein solution); on the other hand, MALDI does not necessarily correlate well with quantity of analyte in the absence of an internal standard.

Using reflectance FTIR spectroscopy, we also examined the effect of salt on protein adsorption to PAA. The presence of 1 M NaOAc decreases amide absorbances due to RNase A adsorption by 90% (adsorption pH of 8.5). At high concentrations, salts are evidently able to compete with RNase A for binding sites. This is consistent with a mechanism of protein adsorption based on electrostatic forces.

To examine whether proteins are removed from PAA films upon exposure to matrix solutions, we first immersed a PAA-film-covered gold wafer in a 1pmol/μL RNase A solution (pH=7) for ~12 h, and then rinsed the wafer with water, dried it with N_2, and measured the IR spectrum of the film. Next, we

covered the entire surface with 5 μL of 20 mg/mL DHB in water. After evaporation of the matrix solution solvent, we rinsed the surface with water, dried it with N_2, and measured a second IR spectrum. The diminution in amide absorbance (>90% decrease) following matrix deposition and rinsing with water showed that most of the protein molecules were removed from the film. These results suggest that the acidic matrix protonates the carboxylate groups in the PAA film, thereby breaking the ionic interactions between PAA and the positively charged proteins, and allowing protein incorporation into the matrix. This is consistent with the pH-dependent adsorption discussed above.

One of the drawbacks of the PAA system described herein is that only positively charged proteins can be effectively captured from salt-containing solutions. However, we can easily derivatize the PAA substrate to produce an amine-terminated polymer film,[11] which would be positively charged at neutral pH (chapter 3). Using amidation or esterification, one could also modify the surface with specific bioreactive groups, such as antibodies, that have specific affinity for antigens in salt-containing mixtures.[12]

IV. Conclusions

Micro-contact printing of HDT SAMs affords rapid formation of patterned MALDI probes that decrease the detection limit and increase reproducibility in MALDI-MS. Grafting of PAA into bare gold spots of patterned SAMs increases

the versatility of this system and allows desalting of sample solutions prior to MALDI. The combination of a patterned surface and a polymer with affinity for charged proteins yields low-fmol detection limits even in the presence of 1 M NaOAc. Reflectance FTIR spectra confirm that protein binding occurs due to electrostatic interactions. These spectra indicate that protein binding is dependent upon the pH of sample solutions as well as on protein and salt concentration.

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Chapter Three: Use of Polymer-Modified MALDI-MS Probes to Improve Analyses of Protein Digests and DNA

I. Introduction:

Several recent reports demonstrate the effectiveness of on-probe sample purification prior to analysis by MALDI-MS.[1-17] In these purification procedures, surface-modified MALDI probes bind specific analytes, while allowing contaminants to be washed away. By reducing problems of signal suppression or adduct formation, such techniques facilitate successful detection of proteins and DNA during analysis of complex mixtures or solutions contaminated with salts, surfactants, or urea. As mentioned in Chapter 1, successful methods for MALDI sample probe modification include adsorption of self-assembled monolayers (SAMs)[3,12] and deposition of polymers (e.g., polyvinylidenedifluoride (PVDF),[11,18-21] nafion,[22,23] and Teflon.[5,24]) or water-insoluble films of matrix.[5,24-28]

Chapter 2 described preparation of polymer-modified surfaces for MALDI probes using a combination of micro-contact printing and polymer grafting.[29] Deposition of poly(acrylic acid) (PAA) in 200-μm diameter gold spots in a hydrophobic monolayer allows simultaneous concentration and purification of protein from salt-contaminated solutions. Self-centering of sample droplets onto the hydrophilic PAA spots affords sample concentration on the small (200-μm diameter) area during solvent evaporation,[30] while the carboxylate groups of PAA selectively bind proteins having net positive charges, even in the presence of 8 M NaOAc. In this chapter, I describe enhancements to the utility of MALDI probes patterned with PAA through further

chemical (modification) elaboration of these polymeric surfaces. Derivatization of PAA can be controlled to produce one of several different surfaces on the MALDI probe to improve the analysis of protein digests and to remove adducts from DNA.

The availability of a variety of probe surfaces can be especially useful in analyzing protein digests. In conventional MALDI-MS of protein digests, many proteolytic peptides cannot be detected because of signal suppression by salt and other contaminants such as urea or guanidine hydrochloride. This leads to low sequence coverage that sometimes makes protein identification difficult. To solve this problem, researchers often resort to HPLC separation of the digest mixture, but this approach sacrifices the high throughput potential usually available in MALDI-MS. To guide development of a more rapid technique that still enhances the number of proteolytic fragments detected during analysis of a protein digest by MALDI-MS, we evaluated sample probe surfaces modified with cationic, anionic, or hydrophobic groups. Each of these surface types yielded a different mass spectrum with the deposition of a given protein digest, followed by rinsing and subsequent addition of matrix. For example, after the analysis of partially digested myoglobin by MALDI-MS, combining data from a patterned PAA surface and a conventional stainless steel (SS) probe yielded 2.4 times more identifiable proteolytic fragments than did results of analysis using only the SS probe. The effect was less dramatic with a larger protein, bovine serum albumin (BSA), but sequence coverage still increased from 61.3 to 74.5% when including data obtained from individual use of several different surface-modified probes.

Use of derivatized PAA films that present polycationic surfaces is also effective

for on-probe decontamination of DNA samples prior to analysis by MALDI-MS. The salt-tolerance level for analysis of DNA samples by MALDI-MS is usually around 100 mM.[31] At higher salt concentrations, both the peak intensity and resolution decrease due to formation of DNA adducts consisting of multiple cations (see chapter 1, Figure 1.3). The addition of ammonium citrate (AC) to the matrix solution helps to solve this problem because the ammonium ions displace the Na^+ or K^+ bound to DNA; the bound NH_4^+ is lost subsequently as neutral ammonia at the ionization/desorption stage.[32-34] However, purification of DNA samples that contain salt concentrations >100 mM is necessary even when AC is added.[31] Smirnov et al. previously showed that pure polyethylenimine (PEI) or poly(pyrolidone) films are suitable for on-probe purification of DNA samples prior to analysis by MALDI-MS.[10] Here, we show that derivatization of patterned PAA films with PEI also yields polycationic surfaces that adsorb DNA. Simple rinsing of the dried sample on the PAA-PEI surface removes salts (800 mM NaOAc), but not DNA; this procedure leads to regeneration of signals from protonated DNA.

II. Experimental Section

A. Materials and solutions

All proteins and chemicals were obtained from Sigma-Aldrich unless noted otherwise. A 24-mer (TTT CAC CCC TCT ATG ACC GCT ACC), 20-mer (AAC CTT GGA ACC TTG GAA CC), 7-mer (TTT TTT T), and 10-mer (AAC CTT GGA A) of DNA were prepared in the Macromolecular Structure Facility at Michigan State

University. A DNA 14-mer (TTG GCC AAT TCC GG) and a DNA 12-mer (CCG GAA TTG GCC) were obtained from Gene Link. Protein or DNA solutions were prepared in deionized H_2O (milli-Q, 18 MΩcm) or 800 mM NaOAc. Gold-coated substrates (200 nm of gold sputtered on 20 nm of Cr on Si(100) wafers) were prepared by Lance Goddard Associates (Foster City, CA).

B. Protein Digestion

Horse heart myoglobin, BSA, and ribonuclease A (RNase A) were digested by trypsin (Promega) according to the procedures provided by the supplier. Briefly, ~20μg of protein were first denatured either by 50 μL 6 M urea in Tris buffer (diluted to 0.6 M urea before addition of trypsin, final volume ~500 μL) or by heating at 65 °C in 50 μL water for 30 min (final volume ~ 500 μL by addition of 50 mM ammonium bicarbonate). If the protein contained disulfide bonds (BSA and RNase A), it was then reduced by 5 μL of 10 mM dithiotheitol (DTT) and alkylated with 20 μL of 100 mM iodoacetamide. All proteins were finally incubated with 0.5-1 μg trypsin at 37°C overnight. The digestion was stopped by addition of acetic acid to achieve a pH of ~3 or storage of the digestion mixture in a -80 °C freezer.

C. Fabrication of patterned substrates

The procedure for preparing patterned hexadecanethiol (HDT) SAMs on gold was reported previously.[29,35] Briefly, we first prepared a polydimethylsiloxane (PDMS) stamp according to the methods developed by Whitesides and coworkers.[36] Wetting

of the PDMS stamp with ~10 mM HDT in ethanol, followed by pressing the stamp onto an ozone-cleaned gold wafer for 30 sec transferred the pattern of the stamp onto the gold surface. After rinsing with ethanol and water, immersion of the patterned wafer in a 1-mM ethanolic solution of mercaptoundecanoic acid (MUA) for 60 s resulted in a patterned MUA/HDT SAM. Activation of the carboxylic acid groups of MUA with ethylchloroformate, grafting of amino-terminated poly(*tert*-butyl acrylate) (PTBA) to these groups, and subsequent hydrolysis in methanesulfonic acid solution yielded hydrophilic PAA[37] spots surrounded by hydrophobic regions of HDT. To modify the patterned surfaces, we activated the PAA with ethylchloroformate again and allowed this surface to react with 20 mg/mL PEI in N,N-dimethylformamide (DMF) for 1 h. Rinsing with ethanol and water removed physisorbed PEI, and subsequent rinsing of the surface with water or dilute HCl protonated the amine groups in PEI, thereby creating hydrophilic, positive spots in the hydrophobic HDT SAM. PEI can also be attached directly to an ethylchloroformate-activated MUA/ HDT SAM to produce a MUA-PEI-modified probe. In an alternative chemical elaboration procedure, we immersed the patterned HDT SAM/ PAA film into an aqueous solution of 100 mM $Fe(NO_3)_3$ or $Fe(ClO_4)_3$ for 15 min to form PAA-Fe^{3+}, complexes and subsequently rinsed the film with water.

D. Sample Preparation prior to Analysis by MALDI-TOF-MS

For analysis of protein digests, four types of surfaces were used: conventional SS, gold modified with hydrophobic HDT SAMs, and gold patterned with HDT SAM/

PAA or HDT SAM/ PAA-PEI films. A droplet of protein digest solution (0.25 μL) was applied to each modified surface and allowed to air dry. Subsequently, the sample area was rinsed with water (~10 mL, from a wash bottle) for about 5 s (to remove interfering contaminants) and dried with N_2. A matrix solution (0.25 to 0.5 μL of 50:50:0.1 v:v:v acetonitrile:water:trifluoroacetic acid saturated with α-cyano-4-hydroxycinnamic acid, α–CHCA) was then added to the spots and allowed to air dry. In the case of the conventional SS surface, no water rinsing was included, and matrix was added before the sample dried. For the analysis of salt-contaminated DNA solutions, a 0.25-μL sample was applied to a PAA-PEI or MUA-PEI spot, air dried, and then rinsed with water from a wash bottle for ~5 s. Subsequently, a 10 to 50 mM AC solution saturated with 3-hydroxypicolinic acid (3-HPA) was used as a comatrix.

E. HPLC Separation of Protein Digests

Aliquots (10 to 50 μL) of myoglobin tryptic digests were injected into an HPLC and subjected to reverse-phase separation with a 5% to 95% solvent B gradient elution in 60 minutes. (Solvent A: 95% H_2O, 5% acetonitrile, 0.1% trifluoroacetic acid; Solvent B: 5% H_2O, 95% acetonitrile, 0.1% trifluoroacetic acid) Each elution fraction (25 to 80μL) was collected, evaporated to 5 to 10 μL with a Speed Vac, and analyzed by MALDI-MS.

G. Instrumentation and Data Analysis

Mass spectra were obtained with a PE Biosystems Voyager STR MALDI TOF mass spectrometer using an accelerating voltage of 20 kV, a 94% grid voltage, a 0.05% guidewire voltage, and an extraction delay time of 100-150 nsec to accumulate ion current associated with 50-250 laser pulses. A 2-point m/z calibration for protein digests was usually performed using intense peaks corresponding to previously identified peptides. Peaks were selected according to the following protocol: the root mean square (RMS) noise level as a percentage of the base peak was first calculated for the whole spectrum, and this value was multiplied by 20 to obtain the percent peak area threshold for peak detection. Signals were assigned to specific peptides using the Mascot program (http://www.matrixscience.com) with a mass tolerance of 1 Da. Peptides corresponding to signals consisting of a small shoulder or a peak with height <3 times the local noise were not counted as peptide matches. (This was the case for fewer than 6% of identified peaks. Molecular weight search (MOWSE) scores were calculated without removing low-intensity peaks.) Ellipsometric determinations of polymer thickness were performed with a rotating analyzer spectroscopic ellipsometer (J.A. Woollam, M-44), assuming a film refractive index of 1.5. Reflectance FTIR spectra were obtained with a Nicolet Magna 560 spectrophotometer using a Pike grazing angle (80°) accessory; the spectrophotometer was housed in a glove box to minimize interference from water vapor.

III. Results and Discussion

A. Analysis of Protein Digests by MALDI-MS

Mass spectra of a tryptic digest of myoglobin show that use of different sample probe surfaces permits the detection of different, and sometimes complementary, arrays of proteolytic fragments. Figure 3.1a shows a MALDI mass spectrum of a tryptic digest of myoglobin that was denatured with 6 M urea and analyzed using a conventional SS probe. The signal at m/z 1608 is particularly strong, and small signals from ten other protonated proteolytic fragments are also visible. The dominating signal at m/z 1608 may arise from selective tryptic cleavage that leads to a high concentration of this peptide, or this fragment may have an especially high ionization or detection efficiency. To gain further insight into the MALDI process, we used HPLC to quantitatively assess the distribution of proteolytic fragments in the tryptic digest of myoglobin. The chromatographic peak (data not shown) representing the tryptic fragment responsible for the mass spectral peak at m/z 1608 was not particularly intense (compared with other peaks), indicating that the concentration of this peptide in the digest mixture was not unusually abundant. This observation suggests that this fragment (m/z 1608, residues 17-31, see Table 3.1 for composition) has a higher ionization or detection efficiency than other components in the digestion mixture. Ionization of this peptide (m/z 1608) may be especially tolerant to the presence of urea (see below).

Table 3.1 Properties of peptides detected with MALDI-MS analysis of a myoglobin tryptic digest using different surfaces.

a. Stainless Steel

Start - End	Mr(expt)	Mr(calc)	Delta Mr	B&B index	Charge	Sequence
32 - 42	1271.38	1271.44	-0.06	-1430	0	LFTGHPETLEK
64 - 77	1378.53	1378.68	-0.15	-3130	2	HGTVVLTALGGILK
119 - 133	1502.66	1502.64	0.02	5540	0	HPGNFGADAQGAMTK
17 - 31	1606.81	1606.8	0.01	770	-1	VEADIAGHGQEVLIR
32 - 45	1661.55	1661.88	-0.33	-1880	0	LFTGHPETLEKFDK
1-16	1815.47	1816	-0.53	60	-1	GLSDGEWQQVLNVWGK
80 - 96	1853.76	1854.06	-0.3	4860	3	GHHEAELKPLAQSHATK
103 - 118	1884.74	1885.19	-0.45	-6940	1	YLEFISDAIIHVLHSK
79 - 96	1981.68	1982.23	-0.55	5320	4	KGHHEAELKPLAQSHATK
78 - 96	2109.771	2110.4	-0.629	5780	5	KKGHHEAELKPLAQSHATK
134 - 153	2282.774	2283.61	-0.836	-2320	0	ALELFRNDIAAKYKELGFQG

In this table, "Start-End" is the starting and ending amino acid residue number in the myoglobin sequence; "Mr(expt)" is the m/z value of the corresponding peptide observed in the MALDI mass spectrum subtracted by 1.0079 (mass of a proton); "Mr(calc)" is the theoretical m/z value predicted for the corresponding peptide; "Delta Mr" is the difference between "Mr(expt)" and "Mr(calc)"; "B&B index" is a hydrophobicity scale calculated by GPMAW software (Perseptive); "Charge" is the total number of charges on the corresponding peptide.

b. Hydrophobic HDT SAM on Au

Start - End	Mr(expt)	Mr(calc)	Delta Mr	B&B index	Charge	Sequence
103 - 118	1885.22	1885.19	0.03	-6940	1	YLEFISDAIIHVLHSK
97 - 118	2602.15	2602.12	0.03	-8400	4	HKIPIKYLEFISDAIIHVLHSK
103-133	3369.62	3369.8	-0.18	-1400	1	YLEFISDAIIHVLHSKHPGDFGADAQGAMTK
1-31	3404.54	3404.79	-0.25	830	-2	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR

c. HDT SAM/ PAA-PEI on Au

Start - End	Mr(expt)	Mr(calc)	Delta Mr	B&B index	Charge	Sequence
64 - 77	1378.71	1378.68	0.03	-3130	2	HGTVVLTALGGILK
64 - 78	1506.86	1506.85	0.01	-2670	3	HGTVVLTALGGILKK
1 - 16	1816.08	1816	0.08	60	-1	GLSDGEWQQVLNVWGK
103 - 118	1885.36	1885.19	0.17	-6940	1	YLEFISDAIIHVLHSK
134 - 153	2283.99	2283.61	0.38	-2320	0	ALELFRNDIAAKYKELGFQG
99 - 118	2337.03	2336.8	0.23	-9550	2	IPIKYLEFISDAIIHVLHSK
97 - 118	2602.14	2602.12	0.02	-8400	4	HKIPIKYLEFISDAIIHVLHSK
48 - 77	3218.57	3218.8	-0.23	-490	3	HLKTEAEMKASEDLKKHGTVVLTALGGILK
103 - 133	3369.19	3369.8	-0.61	-1400	1	YLEFISDAIIHVLHSKHPGDFGADAQGAMTK
1-31	3404.32	3404.79	-0.47	830	-2	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR
99 - 133	3820.62	3821.41	-0.79	-4010	2	IPIKYLEFISDAIIHVLHSKHPGDFGADAQGAMTK

d. HDT SAM/ PAA on Au

Start - End	Mr(expt)	Mr(calc)	Delta Mr	B&B index	Charge	Sequence
64 - 77	1378.7	1378.68	0.02	-3130	2	HGTVVLTALGGILK
64 - 78	1506.86	1506.85	0.01	-2670	3	HGTVVLTALGGILKK
17 - 31	1607.24	1606.8	0.44	770	-1	VEADIAGHGQEVLIR
64 - 79	1635.14	1635.03	0.11	-2210	4	HGTVVLTALGGILKKK
80 - 96	1854.23	1854.06	0.17	2640	1	GHHEAELKPLAQSHATK
103 - 118	1885.19	1885.19	0	-6940	1	YLEFISDAIIHVLHSK
32 - 47	1937.29	1937.23	0.06	-2940	1	LFTGHPETLEKFDKFK
79 - 96	1982.29	1982.23	0.06	5320	4	KGHHEAELKPLAQSHATK
46-62	2005.61	2005.32	0.29	1120	1	FKHLKTEAEMKASEDLK
78 - 96	2110.48	2110.4	0.08	5780	5	KKGHHEAELKPLAQSHATK
46-63	2133.97	2133.49	0.48	1580	2	FKHLKTEAEMKASEDLKK
57-77	2150.54	2150.55	-0.01	-1710	2	ASEDLKKHGTVVLTALGGILK
57-78	2278.53	2278.72	-0.19	-1250	3	ASEDLKKHGTVVLTALGGILKKK
134 - 153	2283.6	2283.61	-0.01	-2320	0	ALELFRNDIAAKYKELGFQG
32 - 50	2315.51	2315.7	-0.19	-3440	3	LFTGHPETLEKFDKFKHLK
99 - 118	2336.87	2336.8	0.07	-9550	2	IPIKYLEFISDAIIHVLHSK
97 - 118	2602.14	2602.12	0.02	-8400	4	HKIPIKYLEFISDAIIHVLHSK
48 - 77	3218.78	3218.8	-0.02	-490	3	HLKTEAEMKASEDLKKHGTVVLTALGGILK
48-78	3346.88	3346.98	-0.1	-30	4	HLKTEAEMKASEDLKKHGTVVLTALGGILKK
103 - 133	3369.6	3369.8	-0.2	-1400	1	YLEFISDAIIHVLHSKHPGDFGADAQGAMTK
1 - 31	3404.71	3404.79	-0.08	830	-2	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR
99 - 133	3820.94	3821.41	-0.47	-4010	2	IPIKYLEFISDAIIHVLHSKHPGDFGADAQGAMTK

Figure 3.1 also presents the MALDI mass spectra of the same tryptic digest of myoglobin deposited on a HDT SAM/ PAA film, a HDT SAM, and a HDT SAM/ PAA-PEI film. The digest mixture was applied to these modified surfaces, allowed to dry, and rinsed with water before adding matrix. The spectra clearly show that the relative signal intensity at m/z 1608 decreases dramatically when using the modified surfaces with the rinsing step. Additionally, signals from a number of previously undetected peptides appear. The diminution of the signal at m/z 1608 possibly occurs because the water rinse removes a large fraction of this relatively hydrophilic peptide (see table 3.1 for peptide sequence). Other peptides that are hydrophobic or capable of electrostatically interacting with a modified surface are less likely to be removed by rinsing.

Table 3.1 lists the tryptic fragments of myoglobin detected from various probes, their amino acid sequences, their net charges at pH 5.3 (~pH of the rinsing solution), and their Bull and Breese (B & B) hydrophobicity indices. Three of the four fragments detected using the hydrophobic HDT SAM have highly negative B & B indices, suggesting that these peptides are primarily retained via hydrophobic interactions. Peptides detected on PAA surfaces generally have either negative B & B indices or they are positively charged, suggesting that both hydrophobic and electrostatic interactions contribute in the binding process. For the PAA-PEI surface, most of the detected peptides have negative B & B indices, and the two peptides with positive B & B indices were negatively charged, suggesting that both hydrophobic and electrostatic interactions were responsible for peptide adsorption.

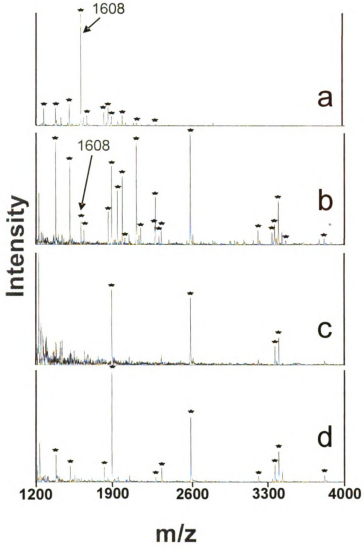

Figure 3.1 MALDI mass spectra of a tryptic digest of myoglobin (500 fmol) deposited on different probe surfaces: (a) conventional stainless steel (b) hydrophilic anionic spots in a hydrophobic field, HDT SAM/ PAA; (c) a hydrophobic surface, HDT SAM; (d) hydrophilic cationic spots in a hydrophobic field, HDT SAM/ PAA-PEI. In (b)-(d), the samples were deposited on the polymer-modified surface and rinsed with water prior to addition of a-CHCA. Stars (★) indicate peaks that can be assigned to tryptic fragments of myoglobin.

Overall, the MALDI mass spectrum obtained using the HDT SAM/ PAA-modified surface (Figure 3.1b) contains signals due to 22 proteolytic fragments of myoglobin, and only seven of these signals were detectable when using the SS sample probe, from which only 11 total tryptic fragments were detected. This increase in the number of tryptic fragments with the PAA-modified surface significantly increased the confidence level in identifying myoglobin. For example, when using the Mascot program to identify myoglobin with data obtained using the SS probe, the probability-based MOWSE score was 82. In contrast, protein identification using data accumulated with the HDT SAM/ PAA-modified surface gave a MOWSE score of 215. Mass spectral data obtained by using the PAA-modified probe greatly decreased the probability (from 0.25% to $10^{-13.9}$%) that the protein identification occurred due to a random match. Interestingly, although analyses from HDT SAM and HDT SAM/ PAA-PEI surfaces yielded 11 proteolytic fragments (Figures 3.1c and 3.1d), all of these signals were also present in the MALDI mass spectra obtained using either the PAA-modified sample surface or the SS probe.

The data in Figure 3.1 were obtained from myoglobin that was denatured with 6 M urea. When myoglobin is denatured at 65 °C before digestion by trypsin, the mass spectrum of the digest from a conventional SS sample plate shows about the same number of tryptic fragment signals as does the spectrum taken using a PAA-modified surface, although relative intensities are quite different. In the case of the SS sample probe, addition of urea (0.6 M) to the heat-denatured digest decreases the number of identifiable proteolytic fragments to the level seen in the spectrum of the digest prepared using preliminary urea denaturation. This suggests that the main advantage of using the polymer-modified surfaces on the sample probe lies in allowing the removal of urea. (Although thermal denaturation avoids urea-contamination of a

protein sample destined for analysis by MALDI-MS, it breaks electrostatic bonds only reversibly and is rarely capable of disrupting the hydrophobic core of most proteins. Thus, 6 M urea continues to be the chaotropic agent of choice in the preliminary preparation of proteins for proteolytic digestion.[1])

Sequence coverage is another figure of merit in protein mass mapping by MALDI-MS. Usually, ~90 to 100% sequence coverage can be achieved for smaller proteins like myoglobin; however, for bigger proteins, such as BSA, sequence coverages are usually low.[2] The conventional MALDI mass spectrum (See Figure 3.2) of an incomplete tryptic digest of BSA (urea denaturation) contains signals from numerous tryptic fragments, resulting in a sequence coverage of 61.3%, or 372 amino acid residues out of 607. With additional MS data obtained using each of the three modified probes (data not shown), sequence coverage increases by 13.2 percent to include another 80 amino acid residues. B. DNA Analysis Using Positively Charged MALDI Probes Patterned HDT SAM/ PAA-PEI-modified surfaces are also useful for on-probe decontamination of DNA samples prior to analysis by MALDI-MS. As a previous study shows,[3] the salt-tolerance level for successful analysis of DNA samples by MALDI-MS is around 100 mM. At higher salt concentration, both mass spectrometric signal intensity and resolution degrade due to formation of multiple-cation adducts of DNA. This problem is apparent in Figure 3.4a, where a broad, low-intensity signal occurs when a 60-fmol sample of a DNA 24-mer in 800 mM NaOAc is analyzed by MALDI-MS from a SS sample probe. However, a sharp peak from the protonated DNA 24-mer (Figure 3.4b) results with use of a PAA-PEI –modified sample probe and a water rinse before analysis by MALDI-MS.

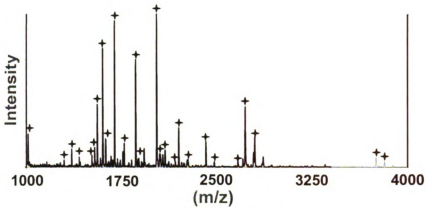

Figure 3.2 MALDI mass spectrum of a BSA (1 pmol) tryptic digests obtained on a SS probe. Stars (★) indicate peaks that can be assigned to tryptic fragments of BSA.

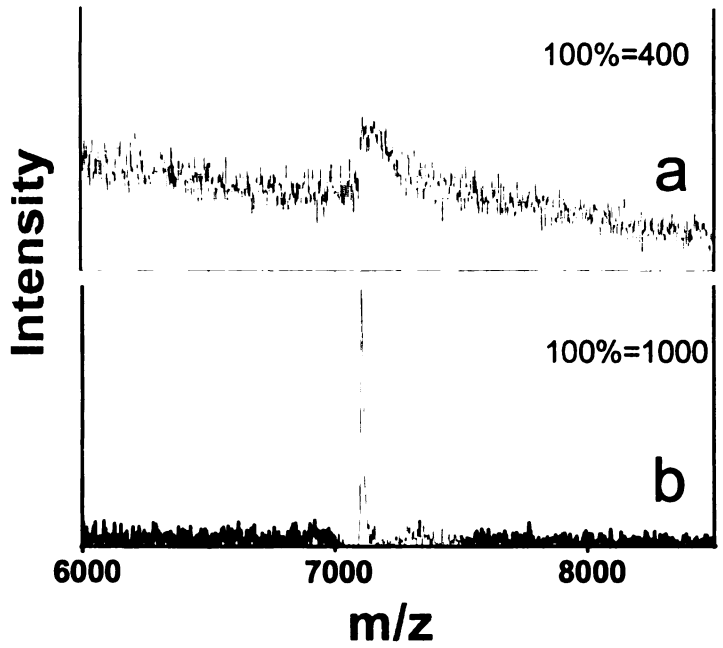

Figure 3.3 MALDI mass spectra of 60 fmol of a DNA 24-mer in 800 mM NaOAc deposited on different probes: (a) SS without rinsing; (b) HDT SAM/ PAA-PEI with rinsing after sample drying. A comatrix consisting of 3-HPA and ammonium citrate was used in both cases.

We note that the DNA sample should be rinsed with H_2O only after it dries; otherwise, the water rinse removes DNA molecules as well as contaminating salts. In contrast, when decontaminating salty *protein* solutions prior to analysis by MALDI, strong signals appear regardless of whether rinsing takes place before or after drying of the sample.[4] Presumably, the difference in behavior between proteins and DNA is related to their different solubilities. Proteins have limited solubility in water, and they tend to bind to each other via hydrophobic interactions. In contrast, DNA molecules are highly soluble in water, so rinsing of a wet sample may break the limited electrostatic bonds between DNA and the PEI surface. However, during the time in which DNA samples are allowed to dry on the probe, the DNA may rearrange around the positive groups of PEI to establish multiple binding interactions that can better withstand water rinsing. The addition of ammonium citrate to the matrix is also necessary in order to observe a MALDI signal from the analyte. The citrate anions probably compete with the bound DNA for the positive sites in PEI, helping to desorb DNA for incorporation into matrix crystals. The ammonium ions should also displace the remaining Na^+ or K^+ adducts, and subsequent elimination of ammonium adducts as NH_3 leaves only the protonated DNA species, which is represented by a well-resolved peak in the MALDI mass spectrum (Figure 3.3b). Similar results can be obtained with a mixture of several DNA strands in 1 M NaOAc. With a mixture of a 7-mer, an 8-mer, a 10-mer, and a 12-mer in 1 M NaOAc, signals are observed for all DNA components in MALDI-MS using MUA-PEI- or PAA-PEI-modified sample probes. (see Figure 3.4, for an example)

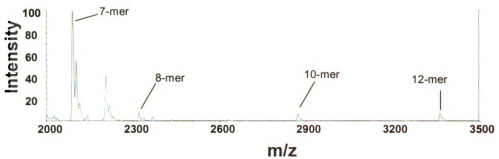

Figure 3.4 MALDI mass spectrum of a DNA mixture contaminated with 1 M NaOAc. The sample was deposited on a modified MUA-PEI surface, air dried, rinsed with water, and subsequently matrix was applied.

IV. Conclusions

Parallel analyses of tryptic digests of proteins by MALDI-MS from sample probes consisting of polymer-modified surfaces containing hydrophobic, cationic, or anionic sites greatly increase the number of peptides that can be detected. The enhancement is likely due to removal of urea after deposition of the sample, and prior to deposition of the MALDI matrix. Polycationic surfaces on MALDI sample probes are also useful in preventing formation of multiple-ion adducts of DNA during analysis of salt-contaminated DNA samples by MALDI-MS, thereby yielding well-resolved peaks for protonated DNA segments.

References:

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(2) Kadlcik, V.; Strohalm, M.; Kodicek, M. *Biochem. and Biophys. Res. Commun.* 2003, *305*, 1091-1093.

(3) Shaler, T. A.; Wickham, J. N.; Sannes, K. A.; Wu, K. J.; Becker, C. H. *Anal. Chem.* 1996, *68*, 576-9.

(4) Xu, Y.; Watson, J. T.; Bruening, M. L. *Anal. Chem.* 2003, *75*, 185-190.

Chapter Four: Use of Polymer-Modified MALDI-MS Probes to Enhance Detection of Phosphopeptides in Phosphoprotein Digest

I. Introduction:

Characterization of protein phosphorylation is essential in proteomics because this posttranslational modification is one of the most important mechanisms for regulating signal transduction, gene expression, and protein synthesis in eukaryotic cells.[1,2] It is estimated that approximately one-third of all proteins in eukaryotic cells are phosphorylated at any given time.[3] However, the analysis of these phosphoproteins is not straightforward due to challenges such as substoichiometric levels of phosphorylation, variation of phosphorylation sites with cell state, and different degrees of phosphorylation at different sites for the same phosphoprotein.[4,5] Traditionally, protein phosphorylation was studied by incorporation of ^{32}P into the cellular proteins via treatment with radio-labeled ATP.[6,7] However, this method suffers from complications due to problems with handling radioactive materials and limited ^{32}P incorporation.[8,9]

In recent years, mass spectrometry (MS) has become an indispensable tool for studying phosphoproteins.[3-5,7,9-24] The detection of phosphopeptides by MS can involve a number of different techniques, including a combination of peptide mapping and phosphatase treatment,[13,24] post-source decay,[10,16,25] precursor ion scanning,[11,21,23] and neutral loss scanning.[12,19] However, the detection of phosphoproteins or phosphopeptides by MS is challenging because signals from these species are often suppressed by non-phosphopeptides in the digest mixture, especially when

phosphorylation occurs at a substoichiometric level. This is clearly shown in Figure 4.1a-c. When pure angiotensin (A, sequence DRVYIHPF, Figure 4.1a) or pure phosphoangiotensin (AP, sequence DRVpYIHPF, Figure 4.1b) was analyzed individually by MALDI-MS, a very strong signal was observed in either case. However, when an equimolar mixture of A and AP was analyzed using MALDI-MS, only signal due to protonated A was observed (Figure 4.1c). Because ionization of phosphopeptides is comparitively weak, several protocols have been designed to enrich phosphopeptides (remove non-phosphorylated peptides) before analysis by MS. For example, antibodies that bind specifically to phosphotyrosine have been used to immunoprecipitate phosphotyrosine(pY)- containing peptides.[26] However, this method is limited to pY-containing peptides or proteins, and cannot be applied to phosphoserine-(pS) or phosphothreonine(pT)-containing peptides. All of the antibodies for pS- and pT-containing peptides found thus far are sequence dependent. Considering the 1800:200:1 ratio of pS:pT:pY found in eukaryotic cells[4,5], this pY-specific antibody method is not particularly useful. A second protocol for enriching phosphopeptides employs reverse phase HPLC to separate non-phosphopeptides from phosphopeptides along with concentration of isolated phophopeptides by solvent evaporation.[12,27] However, multiply-phosphorylated peptides can be quite hydrophilic and co-elute with salts, while less hydrophilic, mono-phosphorylated peptides may co-elute with the corresponding non-phosphorylated peptides, making HPLC separation futile. Adsorption of peptides to vial walls during evaporation and handling can be a serious secondary

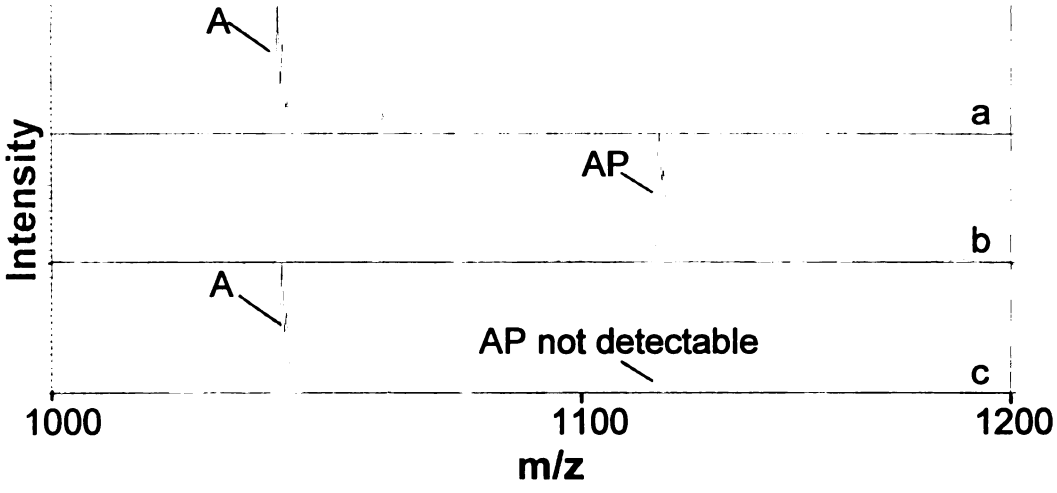

Figure 4.1 MALDI mass spectra of: (a) 5 pmol Angiotensin (A); (b) 5 pmol Phosphorylated Angiotensin (AP); (c) 2.5 pmol of A and AP. α-CHCA was used as matrix in every case.

problem that reduces sensitivity.

Recently, immobilized metal affinity chromatography (IMAC) has become popular for the enrichment of phosphopeptides prior to MS analysis. This technique utilizes the complexation of phosphate groups by metal ions to retain phosphopeptides, while other peptides are removed during washing.[28-30] However, nonspecific binding of peptides containing multiple acidic residues and difficulty in eluting multiply phosphorylated peptides can be problematic in IMAC. Thus, proper use of this technique requires that the analyst has a thorough understanding of the operating principles of the separation system. IMAC beads carrying adsorbed phosphopeptides also have been analyzed directly on MALDI probes following multiple rinsing with dilute acetic acid solutions.[9,17,29] Compared with the traditional IMAC technique, this method avoids the time-consuming HPLC process. Deposition of these beads followed by addition of matrix solution is sufficient to observe signals due to mono-phosphopeptides. However, in order to desorb multi-phosphorylated peptides from beads, addition of 100mM ammonium dihydrogenphosphate to these beads is necessary.[29]

In this chapter, I discuss the use of patterned cationic surfaces on MALDI probes to selectively bind phosphopeptides to enhance their detectabilities. During such experiments, peptide mixtures containing both phospho- and nonphospho-peptides are applied to the cationic surfaces, dried, and then rinsed with water to remove most of the nonphosphopeptides. Surface bound phosphopeptides are released by the subsequently added strongly acidic matrix solutions for their incorporation into matrix

crystals. This technique offers potential advantages over HPLC and traditional IMAC in that it allows for rapid sample preparation and avoids loss of peptide on vial walls.

II. Experimental Section

A. Materials and solutions

All proteins and chemicals were obtained from Sigma unless noted otherwise. Protein solutions were prepared in deionized H_2O (milli-Q, 18 MΩcm). Three peptides, angiotensin (A), phosphoangiotensin (AP) and another phosphopeptide with unknown sequence (UP, m/z 1858) were kindly provided by Rhonda Husain from the Mass Spectrometry Facility at Michigan State University. Mixed linkage kinase 3 (MLK3) was kindly provided by Professor Gallo of Michigan State University and prepared by Karen Alexandra Schachter. A peptide mixture simulating a phosphoprotein digest was prepared by mixing A, AP, and UP (~5 μM final concentration of each species) with a myoglobin (~1 μM) tryptic digest. Gold-coated substrates (200 nm of gold sputtered on 20 nm of Cr on Si(100) wafers) were prepared by Lance Goddard Associates (Foster City, CA).

B. Protein Digestion and Peptide Dephosphorylation

Horse heart myoglobin, β-casein and ovalbumin were digested by trypsin (promega) according to the procedures provided by the supplier. Briefly, ~5-20 μg of protein was first denatured either by 50 μL of 6 M urea in Tris buffer (diluted to 0.6 M urea before addition of trypsin, final volume ~500 μL) or by heating at 65 °C in 50 μL

water for 30 min (final volume ~ 500 μL by addition of 50 mM ammonium bicarbonate). If the protein contained disulfide bonds (β-casein and ovalbumin), it was then reduced by 5 μL of 10 mM dithiothreitol (DTT) and alkylated with 10 μL of 100 mM iodoacetamide. All proteins were finally incubated with 0.5-1 μg trypsin at 37 °C overnight. In most cases, the digestion was stopped by addition of acetic acid to achieve a pH of ~3. Digested ovalbumin was also incubated with 10 U (enzyme activity unit) phosphatase (New England Biolabs) at 37 °C for 3 hours in a pH 7.5 tris buffer to remove phosphate groups.

C. Fabrication of Patterned Substrates

The procedure for preparing patterned PAA-PEI or MUA-PEI was discussed in chapters 2 and 3. I report here the preparation of another patterned cationic surface, PAA-Fe^{3+}, from patterned anionic PAA. This surface was designed to be somewhat similar to stationary phases used for IMAC. A slide coated with patterned PAA was immersed into a 100 mM $FeCl_3$ aqueous solution or a 100 mM $Fe(ClO_4)_3$ ethanolic solution for 30 minutes, followed by water or ethanol rinsing to remove unattached Fe^{3+}.

D. Sample Preparation prior to Analysis by MALDI-TOF-MS

Sample solution (0.25 μL) was applied to the PAA-PEI or PAA-Fe^{3+} surface, allowed to dry, and then rinsed with water (pH ~5) or dilute acetic acid (100 mM, pH~4). Matrix solution (0.25 μL saturated α-CHCA, in 50:50:0.1 acetonitrile:water:triacetic acid) was applied after drying the water-rinsed surface with

N_2. Finally, the wafer carrying the sample was attached to a disposable MALDI plate using superglue, and the sample was analyzed by MALDI-TOF-MS.

E. Instrumentation and Data Analysis

Mass spectra were obtained with a PE Biosystems Voyager STR MALDI TOF mass spectrometer using an accelerating voltage of 20 kV, a 94% grid voltage, a 0.05 % guidewire voltage, and an extraction delay time of 100-150 nsec to accumulate ion current associated with 50-250 laser pulses. A 2-point calibration for protein digests was usually performed using intense peaks corresponding to previously identified peptides. Peaks were assigned to protonated phosphopeptides by the presence of a m/z – 80 peak (loss of HPO_3), or the disappearance of a particular signal after treatment of the same sample with phosphatase. Ellipsometric determinations of polymer thickness were performed with a rotating analyzer spectroscopic ellipsometer (J.A. Woollam, M-44), assuming a film refractive index of 1.5. Reflectance FTIR spectra were obtained with a Nicolet Magna 560 spectrophotometer using a Pike grazing angle (80°) accessory; the spectrophotometer was housed in a glove box to minimize interference from water vapor.

III. Results and Discussion

A. Simple Mixture of Phosphopeptides

To study the effectiveness of patterned, cationic surfaces for the analysis of phosphopeptides, I first obtained the MALDI mass spectrum of a simple mixture of equimolar A and AP. The results are shown in Figure 4.2. When this sample was

analyzed using a conventional stainless steel plate, only signal due to A was observed, even though the same amounts of A and AP were present in the mixture. However, when analyzing the same sample using a PAA-Fe^{3+}-modified surface and a water rinse after sample deposition, signal due to AP appeared. This result was exciting, as it showed that a simple water rinse helped to solve the suppression problem; but it was not as ideal as we had hoped it would be. Ideally, the water rinse should remove most of the nonphosphopeptide while the phosphopeptide remains bound to the surface via electrostatic interactions. In the mass spectrum in Figure 4.2b, the peak due to the nonphosphopeptide is still the dominant peak, suggesting that a good portion of this peptide remains on the surface after the water rinse. These results suggest that the modified cationic surface has higher affinity to phosphopeptides than to non-phosphopeptides, but the selectivity still needs to be improved in order to totally separate nonphosphopeptides from phosphopeptides, and thus completely eliminate the suppression problem.

B. Simulated Phosphoprotein Digest

In order to obtain a more complicated phosphopeptide-containing mixture that represents a typical tryptic digest of a phosphoprotein, I intentionally mixed three peptides, A, AP, and UP, with a tryptic digest of myoglobin. As shown in Figure 4.3, when the mixture was analyzed on a SS probe, signals due to the two phosphopeptides, AP and UP, were hardly observable. However, when the same sample was applied to a PAA-PEI-modified probe and rinsed with water, signals due to AP and UP were

greatly enhanced, and signals due to the tryptic peptides from myoglobin decreased dramatically. Interestingly, the peak that is due to A is still the dominant peak, suggesting that this peptide has a stronger affinity to PAA-PEI than other non-phosphopeptides in the myoglobin tryptic digest mixture.

C. Tryptic Digest of Phosphoproteins

Analysis of ovalbumin by MALDI-MS further illustrates the challenge in detecting phosphorylated fragments using a SS probe. Figure 4.4a shows a MALDI mass spectrum of a tryptic digest of ovalbumin loaded onto a conventional SS sample probe. Some of the peaks that represent phosphorylated fragments (peaks labeled with "P", m/z 2091, EVVGpSAEAGVDAASVSEEFR, m/z 2513, LPGFGDpSIEAQCGTSVNVHSSLR, and m/z 2903, FDKLPGFGDpSIEAQCGTSVNVHSSLR) show low signal intensities. I assigned these peaks to phosphorylated tryptic fragments based on the sequence of ovalbumin and the presence of accompanying peaks corresponding to MH^+ – 80 Da (loss of HPO_3). My attempt to use LC-MS to identify phosphorylated peptides failed, presumably because other peptides co-eluted with the phosphorylated proteolytic fragments. Use of both PAA-Fe^{3+} and PAA-PEI probes (Figure 4.4b and c) resulted in a greatly increased intensity for the phosphorylated fragment at m/z 2091. For PAA-PEI, we attribute these results to binding of anionic, phosphorylated peptides to the polycationic surface of the modified MALDI probe; while in the case of patterned

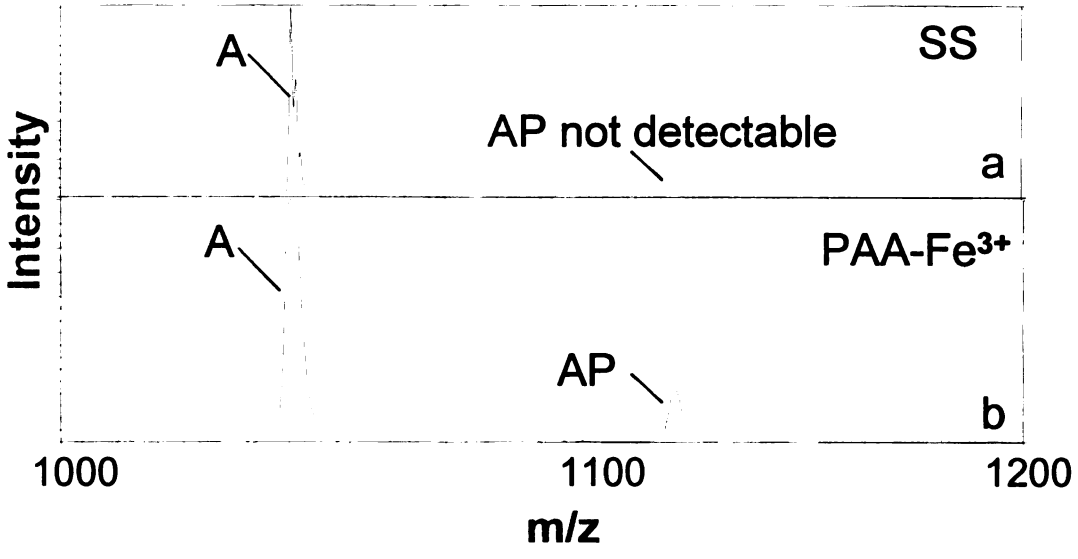

Figure 4.2 MALDI mass spectrum of: (a) Equimolar mixture of angiotensin (A) and phosphoangiotensin (AP), 2.5 pmol each, analyzed using stainless steel probe; (b) the same sample as in (a), applied to a PAA-Fe^{3+} modified probe, dried, and rinsed with water. Saturated α-CHCA was used as matrix in both cases.

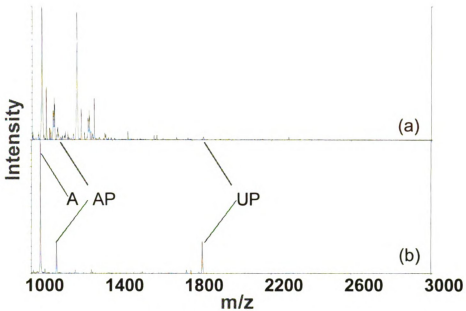

Figure 4.3 MALDI mass spectra of a peptide mixture prepared by mixing two phosphopeptides, "AP" and "UP", and a non-phosphopeptide, "A", 5 μM each, with a tryptic digest of myoglobin. (a)The sample was analyzed on a SS probe; (b) The sample was analyzed with a patterned, PAA-PEI-modified probe. Saturated α-CHCA was used as matrix in both cases.

PAA-Fe^{3+}, the phosphate groups might reversibly form complexes with Fe^{3+}. The IMAC technique (including use of IMAC beads directly on the probe) also relies on binding through metal ion/ phosphate complexes,[22,28-31] but that technique is not as simple as use of polymer-modified MALDI sample probes.

To verify that the labeled peaks (p) in Figure 4.4 represented phosphorylated fragments, I incubated an aliquot of the same tryptic digest of ovalbumin with phosphatase, an enzyme that selectively removes phosphate groups. After 3 hours of incubation at 37 °C, I analyzed this phosphatase-treated digestion mixture using a SS probe and the cationic PAA-Fe^{3+} surface. The MALDI mass spectra (Figure 4.4d and 4.4e) showed no detectable peaks for the phosphorylated peptides seen in Figures 4.4b and c, and corresponding nonphosphorylated peptide peak intensities increased. Similar results were obtained for a β-casein digest, where signals due to two phosphopeptides (m/z 2063.0, FQpSEEQQQTEDELQDK and m/z 3123.9, RELEELNVPGEIVEpSLpSpSpSEESITR) were selectively enhanced as shown in Figure 4.5. It should also be noted that both of these peptides are highly negatively charged, and the acidic amino acid residues may also contribute to the binding process.

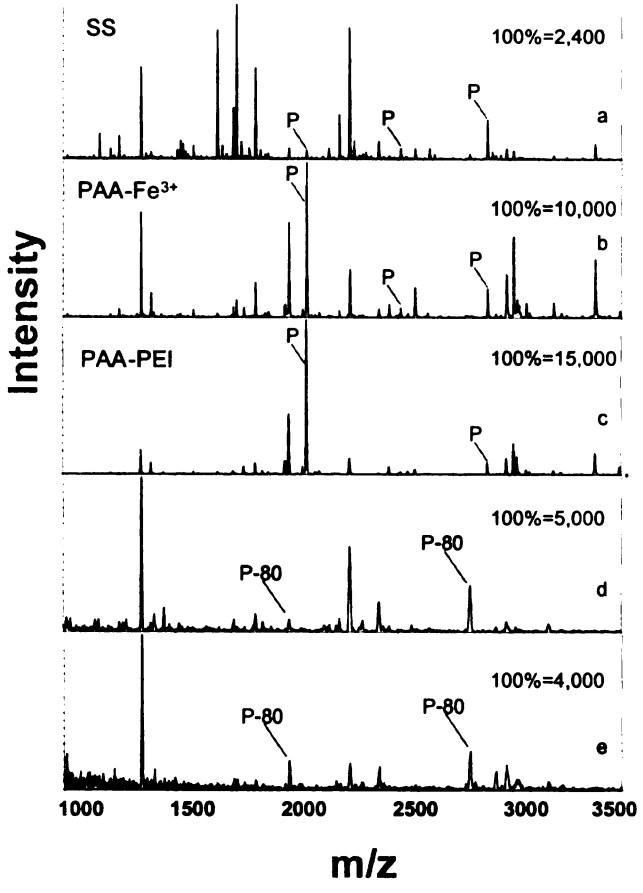

Figure 4.4 Mass spectra of a tryptic digest of ovalbumin (1 pmol) obtained using different MALDI probes: (a) conventional SS; (b) PAA-Fe3+; (c) PAA-PEI; (d) after phosphatase treatment and analyzed on SS; (e) after phosphatase treatment and analyzed on PAA-Fe3+. In (b), (c) and (e), samples were deposited on the probe and rinsed with water prior to addition of matrix. Peaks labeled with "P" represent phosphorylated peptides and peaks labeled with "P-80" represent corresponding nonphosphorylated peptides.

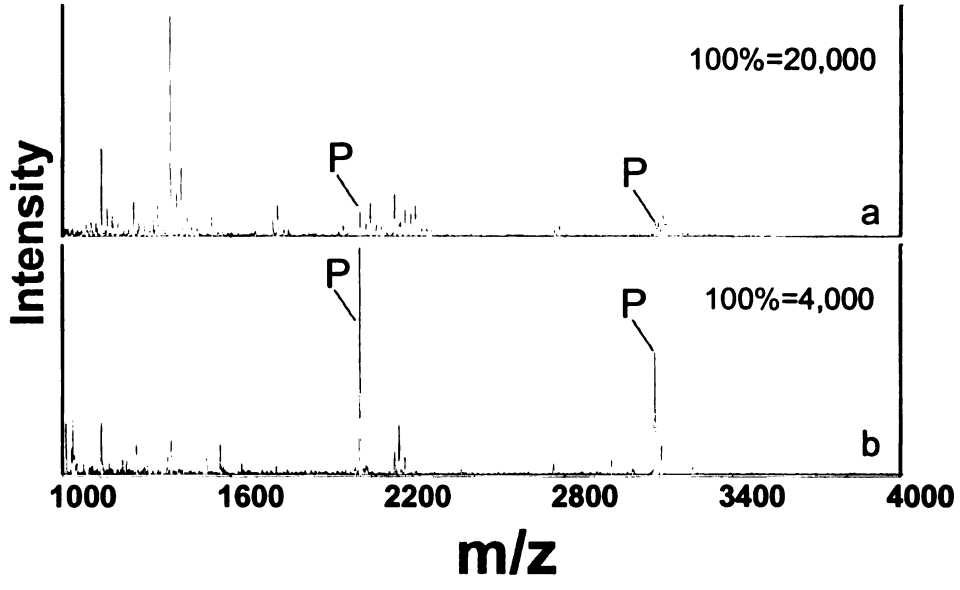

Figure 4.5. Mass spectra of a tryptic digest of b-casein (100 fmol) obtained using different MALDI probes: (a) conventional SS; (b) HDT SAM/ MUA-PEI. In (b), the sample was deposited on the probe and rinsed with water prior to addition of matrix. The peaks labeled with "P" represent phosphopeptides.

IV Conclusion

Detection of phosphorylated peptides during MALDI-MS of digests deposited on polycationic surfaces consisting of PAA-PEI or PAA-Fe^{3+} is particularly attractive because these analytes are often undetectable when using conventional SS sample probes. The enhanced detectabilities for phosphopeptides are probably due to the removal of interfering non-phosphopeptides, which reduces suppression.

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Chapter Five: Conclusions and Future Work

I. Conclusions:

Microcontact printing of HDT SAMs affords rapid formation of patterned MALDI probes that decrease detection limits and increase reproducibility in MALDI-MS. Grafting of PAA into bare gold spots of patterned SAMs increases the versatility of this system and allows desalting of sample solutions prior to analyses by MALDI. The combination of patterning and surface modification with a polymer that binds charged proteins yields low-femtomole detection limits, even for samples containing 1M NaAc. Reflectance FT-IR spectra confirm that protein binding occurs due to electrostatic interactions and, thus, is highly dependent upon the pH of sample solutions. Protein binding to the polymer-modified surface also depends on protein and salt concentration.

Patterned surfaces also show great promise for the analysis of more complicated mixtures, such as protein digests. Parallel analyses of tryptic digests of proteins by MALDI-MS from sample probes consisting of polymer-modified surfaces containing hydrophobic, cationic, or anionic sites greatly increase the number of peptides that can be detected. The enhancement is likely due to removal of urea after deposition of the sample, and prior to deposition of the MALDI matrix. Detection of phosphorylated peptides during MALDI-MS of digests deposited on polycationic surfaces consisting of PAA-PEI or PAA-Fe^{3+} is particularly attractive because these analytes are often undetectable when using conventional SS sample probes. Polycationic surfaces on MALDI sample probes are also useful in preventing formation of multiple-ion adducts

of DNA during analysis of salt-contaminated DNA samples by MALDI-MS, thereby yielding well-resolved peaks for protonated DNA segments.

These examples illustrate the utility of patterned surfaces as MALDI probes, but there are many other areas in which patterned probes may prove useful. Below, I describe one of these areas along with possible improvements in the design and use of patterned probes.

II. Future Work

1. Catch and Release of Free Cysteine-Containing Peptides Using Au

Shotgun proteomics uses trypsin to digest the whole proteome and multi-dimensional liquid chromatography to separate the resulting complicated peptide mixture to reduce ionization suppression problems during subsequent analyses by MALDI-MS.[1-7] However, due to the complexity of the mixture, it is rarely possible to totally separate all the peptides from one another; current approaches aim at simplifying the mixture rather than total separation. We propose to use bare Au surfaces to catch and release free cysteine-containing peptides to simplify complex peptide mixtures in those cases where interest is focused on this specific class of peptides.

The formation of SAMs of alkanethiols on gold has been thoroughly studied,[8] but the study of cysteinyl peptide adsorption to Au is fairly new.[9] Kirk and Bohn recently used a short peptide containing an N-terminal cysteine as a model for cysteinyl peptide adsorption. Matrix was added to the Au colloids carrying the adsorbed peptides, but

only weak signals due to the peptide were observed in the subsequent MALDI experiment. Weak signals probably occurred because the MALDI matrix is not strong enough to remove (cleave) the majority of the adsorbed peptides from Au. Preliminary experiments show that dilute I_2 solutions can remove alkanethiol SAMs from the Au surface.[10] Thus, addition of I_2 to the matrix solution may allow stronger signals to be observed for similar experiments with peptides adsorbed to Au.

2. PAA-nitrilotriacetic acid-Fe^{3+} Surfaces for Enhanced Capture of Phosphopeptides

Use of PAA-Fe^{3+} surfaces to selectively adsorb phosphopeptides on MALDI probes shows some promise in increasing the detectability of phosphopeptides. However, the non-specific adsorption of acidic amino acid residues containing carboxylic acid groups decreases the selectivity of this surface. Use of more stringent washing with a low pH solution may increase selectivity for phosphopeptide adsorption, but Fe^{3+} ions may also be removed from the surface during washing because PAA is not a very strong chelator of Fe^{3+}. In contrast, due to its tetradentate nature, nitrilotriacetic acid (NTA) is a much stronger chelating agent for Fe^{3+} and thus should withstand stringent rinsing. Derivatization of PAA with NTA followed by the formation of a PAA-NTA-Fe^{3+} complex, should result in films that more selectively adsorb phosphopeptides.

3. Patterned Polymer Brushes

A low binding capacity due to the limited thickness of PAA films may lead to the low signal intensities in MALDI-MS experiments using these polymer-modified sample probes. Thus, it may be necessary to grow thicker films to improve binding capacities of these surfaces. Our current methodology uses only one cycle of PAA deposition to obtain a film thickness of ~20 Å. More cycles of derivatization with PAA may create thicker films, but the hydrophobic/ hydrophilic pattern may be compromised due to physisorption of PTBA to the hydrophobic HDT SAMs. Recently, several methods have been developed for creating thicker, patterned films by growth of polymer bruses.[11-14] By combining our expertise in MALDI sample probe modification with established techniques for growing polymer brushes, we should be able to increase the binding capacity of modified surfaces and observe stronger signal intensities in the corresponding mass spectra.

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