Reactive and Soft Landing of Polyatomic Gas-Phase Ions on Plasma-Treated Metal Surfaces

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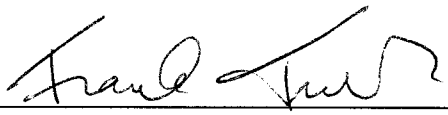

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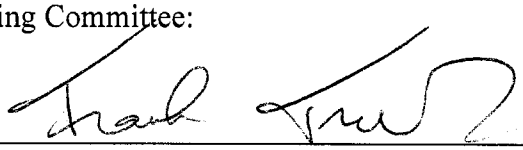

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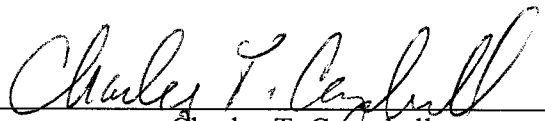

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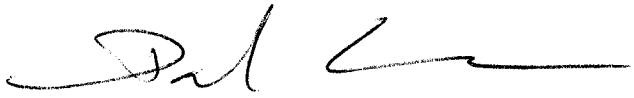

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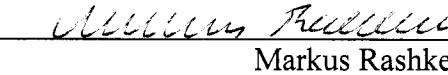

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Abstract

Reactive and Soft Landing of Polyatomic Gas-Phase Ions on Plasma-Treated Metal Surfaces

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Non-destructive physical isolation of ions from the gas phase with preservation of their structure is referred to as ion *soft landing*. The presented dissertation describes *in-situ* plasma oxidized dry metal surfaces as novel and advantageous collection targets (plates) for soft landing experiments. A new term, *reactive landing*, is introduced to cover ion-surface collisions that result in surface immobilization of the impacting ion but without significant damage.

Soft and reactive landing on a plasma-treated metal surface of multiply protonated protein ions results in a substantial retention of protein function, as demonstrated for trypsin and streptavidin. The majority of trypsin ions soft-landed at hyperthermal kinetic energies retain enzymatic activity. The landed streptavidin can be washed into solution where they show affinity to biotin. The layer of streptavidin monomer that is immobilized on the surface can be detected if fluorescence-tagged and retains the ability to reversibly bind biotin. A mechanism is proposed to explain nondestructive protein ion discharge on the surface that considers proton migration from the soft-landed cations to the metal oxide layer and metal ion reduction by electron transfer from the bulk metal.

Soft landing of singly charged gas-phase ions on dry metal surfaces that were pretreated by plasma results in 0.1-6% total yields of recovered intact compounds. The plasma-treated metal surfaces are shown to be useful for preparative separation of organic and biological molecules by mass spectrometry. The instrumental improvement and conditions for reactive immobilization of small molecules are discussed.

The surface-enhanced Raman spectroscopy (SERS) was used to gain structure-specific bonding information for polyatomic cations and molecules soft-landed on plasma-treated silver substrates. While enhancing Raman scattering 10^5-10^6 fold, the metal surface effectively quenches the fluorescence that does not interfere with the Raman spectra. The spectra from zeptomole amounts of soft-landed compounds were found sufficiently intense and reproducible to allow identification of Raman active vibrational modes for structure assignment.

Research results that open new paths to potential applications of soft and reactive landing are described in the last chapter. They include modification of surfaces for biomedical purposes as well as development of protein arrays and modification of CMOS chips.

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GLOSSARY

AA	adjacent-averaging
AFM	atomic force microscopy
APL	Applied Physics Laboratory
B3LYP	Becke's hybrid functional
BAEE	benzoyl-arginine ethyl ester (defines standard unit of trypsin activity)
CCD	charge coupled device
CMOS	complementary metal oxide semiconductor
CRM	charge residue model
CV^+	cation of crystal violet (hexamethylpararosaniline chloride)
DC	direct current
DFPase	diisopropyl-fluorophosphatase
DIPEA	diisopropylethylamine
DMF	dimethylformamide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ESCA	electron spectroscopy for chemical analysis (alternative for XPS)
ESI	electrospray ionization
ESI-MS	mass spectrometry with electrospray ionization
ESI-MS/MS	tandem mass spectrometry with electrospray ionization
GB	gas-phase basicity
HA	hyaluronic acid
HPLC	high-performance liquid chromatography
ID	inside diameter
IEM	ion evaporation model
MALDI	matrix-assisted laser desorption ionization
MP2	Møller-Plesset perturbation theory of the second order
MS	mass spectrometry
m/z	mass to charge ratio
NBD-F	4-fluoro-7-nitrobenzofurazan
OD	outside diameter
OPA	organophosphorus acid
PBS	phosphate buffer solution
PCM	polarizable continuum model
PCR	polymerase chain reaction
PRP	platelet rich plasma
PTE	phosphotriesterase
RF	radiofrequency
SAM	self-assembled monolayer

SEM	scanning electron microscopy
SERS	surface-enhanced Raman scattering (or spectroscopy)
SID	surface induced dissociation
TCN	Tureček Conference Night
UV	ultraviolet
UWEB	University of Washington Engineered Biomaterials
VLK	Valine-Leucine-Lysine (tripeptide)
VVL	Valine-Valine-Lysine (tripeptide)
XPS	X-ray photoelectron spectroscopy

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I truly and gratefully thank the state of Washington and its citizens for accepting me as a guest in this beautiful corner of this great country. In particular I am grateful to the Gudiksen family for their fellowship, awarded to me in 2005. It gave me independence, allowed me to focus on my work, and helped me to complete the most important part of my research project.

There are so many people to thank. First and foremost, I would like to thank František "Frank" Tureček who served as my research advisor. His extensive contribution to this work and helpful insight into my research is gratefully acknowledged. I respect him deeply for his scholastic achievements in many areas of molecular science ranging from theoretical and synthetic chemistry over the analytical mass spectrometry and proteomics to the development of new concepts for vacuum and analytical instrumentation. I really wish that our mutual *Alma Mater*, Charles University in Prague, would one day decide to hire chemistry professor like Frank Tureček. Besides our professional interactions I owe Frank for helping me to discover the Northwest region and for climbing several beautiful mountains together. Climbing with professor Tureček is always and adventure and one never knows what the end of the day will bring or where one will sleep. Frank has widely diverse, almost renaissance, interests and enjoys music, art, foreign cultures and life in general. As an example, it was he who personally demonstrated to me the art of enjoying conference participation at the 52nd ASMS conference in Nashville. To honor Frank, I now have to include one "Tureček Conference Night" (TCN) at every mass spectrometry conference that I attend. Surprisingly, the organizers always schedule the most complex scientific programs on the morning after TCN…

W. Tim Elam, who served as my second and unofficial research supervisor, is not only a brilliant surface physicist but also is an excellent mentor, project manager and person. Tim, father of two little kids, was coordinating our research project between three different UW departments and a private corporation along with his regular work at the Applied Physics Laboratory. He survived this without a major mental damage! His ability to deal with the academic red tape and unexpected problems cannot be called anything less than miraculous. Together with his other skills and abilities, this makes him a truly great project leader and coordinator. Tim built the original soft landing instrument on a nickel-and-dime industrial funding

before I joined the project. I am still amazed by his ingeniously resourceful and economical approach to instrument design. Especially if I consider that he did not come to UW from Eastern Europe but from the U.S. Naval Research Laboratory. I really hope that the US Navy ships are fixed with something little bit more sophisticated than with cork stoppers.

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of us from the very probable electric chair, firing squad, or whatever they use down there. I hope that she still carries that funny Massachusetts Liqueur ID, which created more negative attention when they seized us, than the Czech accent, which surprisingly enough, was taken positively. There certainly are funny moments in graduate school...and *you betcha* that Mrs. Olivová and I will never mess with Texas again.

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DEDICATION

This dissertation is dedicated to my parents Zdeněk Volný and Zdeňka Volná, my sister Kristina Volná and my wife Petra Volná. Without their undying love and support, my successful work would have not been possible, nor would it have meant very much. I cannot even begin to find the words to adequately thank you for your patience, encouragement and sense of humor.

CHAPTER 1: INTRODUCTION

1.1 Prelude to Soft Landing of ions: Lawrence, Calutron, and the Manhattan Project

Deposition of materials following their transport as gas-phase ions has a long history. In the early 1940s, E. O. Lawrence developed a mass spectrometry-based separation approach to enrich ^{235}U from the natural isotopic distribution of uranium. This method used so called Calutrons (the name is a famous concatenation[1]) to separate and collect ions on surfaces according to their mass-to-charge ratio (m/z). As a key part of the Manhattan Project, Lawrence applied this preparative mass spectrometry approach to the purification of ^{235}U.[2–4]

The preparative mass spectrometry (MS) approach was abandoned after World War II because other technologies were offering alternative ways to obtain sufficient quantities of fissionable material. Furthermore, the previous decades had experienced extensive development of separation techniques, involving various principles, which fulfilled many needs in chemistry. For example, chromatography, the most important technique for both analytical and preparative separations, uses differences in polarity to separate different species. The quality of separation thus depends on the ability to distinguish polarity of molecules irrespective of their molecular mass. There are, however, some separation methods that consider mass and/or size as an important factor for the separation process.[5]

The detailed history of Calutrons (including the very few applications that stayed even after the Manhattan project was over and the enrichment of uranium in Calutrons had become an obsolete technique[6]) is summarized in the historical review published in the *Journal of the American Society for Mass Spectrometry* in 1997.[4]

1.2 Soft Landing –extraction of ions from the gas phase

With the advent of non-destructive ionization methods – electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) – it has become possible to nondestructively ionize large polar molecules.

In several reported attempts, non-destructive ionization has been used for physical isolation of ions from the gas phase with preservation of their structure and biological activity (if they had any). These experiments are usually referred to as ion soft landing as first used by Cooks[7] in 1977. This term was also adopted in this PhD thesis. It refers to depositing gas-phase ions at hyperthermal energies on a suitable surface. The soft landed ions can be washed or otherwise recovered from the surfaces back into the solution, analyzed by various analytical techniques and possibly tested for bioactivity.

The goal of my research was to investigate interactions of ions generated by electrospray with plasma oxidized metallic surfaces that now represent a novel category of targets suitable for nondestructive extraction of ions from the gas-phase (soft and reactive landing). These new soft-landing substrates are easier to prepare and handle compare to the previously used Self-Assembled Monolayers (SAM) or liquid surfaces. The plasma oxidation is a necessary step that creates dielectric oxide layer (analogical to, for instance, SAM) that protects the landed ion from direct neutralization by electron transfer, with its subsequent fragmentation and rearrangement.

As was stated above, during soft landing gas phase ions are allowed to strike a surface at kinetic energies typically in the range of 1 eV to several hundreds eV.[7,8] Achieving hyperthermal ion-surface collisions mandates working at pressures where the mean free path of the ion is longer than the ion flight path in the acceleration region between the landing surface and the counter-electrode. The term soft landing usually implies that the ions are not damaged upon collision with the surface. This definition does not distinguish ions that land on the surface with retention of charge or those that undergo discharge. It was already pointed out that a prerequisite for

nondestructive landing of biomolecular ions is that they are produced by a soft ionization. Of these, electrospray ionization offers the advantage of being a continuous ionization method that can provide large ion currents (nA) [9, 10] and hence a substantial mass flow of the analyte to be soft-landed in the vacuum system. One issue of concern is that the gas phase ions, typically even electron ions produced by proton or alkali metal ion attachment, must be discharged upon soft landing to prevent charge build up and deflection of the low-energy ion beam. The mechanism of ion discharge is currently unknown[11, 12] and for the case of oxidized metallic surfaces will be proposed in this doctoral dissertation.

It has been postulated that ions soft landed on surfaces covered with self-assembled monolayers can retain charge for long times and after exposure to the atmosphere[13] although other researchers who are using SAM as landing substrates are expressing doubts about it. [14]

Previous studies of soft landing of polyatomic ions pursued different scientific goals, such as ion implantation,[15-19] surface modification with inorganic clusters,[20-24] ion scattering,[25-42] and surface-induced dissociation (SID).[43-50]

Most relevant for the work presented in this thesis were the recent studies aimed at non-destructive landing of organic cations on surfaces, followed by recovery or analysis of the soft landed material.[51-62] In addition to proving the principle that gas-phase ions can be recovered from the vacuum system of the mass spectrometer, some of the recent studies brought new results of successful recovery of functional biopolymers, such as the polysaccharide hyaluronic acid,[56] several proteins,[57-60] and even intact viruses.[51,52] The largest molecule that was successfully ionized and transported through the vacuum system and landed on a surface was the tobacco mosaic virus. That was found to retain its activity after reconstitution in solution.[51] In another important experiment the DNA standard for polymerase chain reaction (PCR) was soft landed in attomole quantities on a nitrocellulose membrane that was placed inside an ion-cyclotron resonance cell. After the landing the DNA fragment was found to be partially intact and it could be amplified by PCR.[54]

Because of increasing requirements of the biomedical sciences there has been recent interest in using gas phase ions as a vehicle for separation of molecules with important biological relevancy. The new results raised the possibility of using mass spectrometry for preparative purposes for small molecules of pharmaceutical interest[61,62,63] as well as for large biopolymers.[57-60]

1.3 Reactive landing

Interactions of hyperthermal ions with surfaces that result in formation of new bonds on surfaces form the basis of several important industrial processes that result in surface modification, e.g., ion epitaxy[64], ion implantation[65], thin film oxide and nitride deposition[66], and etching[67]. The description of other processes can be found in a review.[24] The fundamental reactive molecule/surface processes are also summarized on Figure 1.1.

During my graduate research work, we have introduced the term "*reactive landing*" (an analogy of soft landing) to describe ion-surface collisions that result in surface immobilization of the impacting ion but without significant damage.[60,68] Thus, reactive landing can be understood as a special example of soft landing. In this work, the ion is considered reactively landed if the bond between the landed molecule and the surface resists washing with polar solvents (e.g. methanol, water or salt solution). As opposed to this, soft landed ions are easily washable by common solvents.

Besides interest in soft and reactive landing due to the separation potential and obvious unique potential for achiving special surface modification, there is a growing opinion that reactive landing could be mainly beneficial for preparation of protein arrays that are less robust than DNA arrays when prepared by current techniques.[69]

Recently, multiply charged enzymes were produced by electrospray and landed in a liquid matrix previously deposited on the landing surface. The enzymes were found to retain partial activity when purified by dialysis and reconstituted in

solution.[57] These promising results showed that large biomolecules can be soft landed and recovered with retention of full or partial activity. Nevertheless, they also opened a number of questions and concerns that need to be solved in order to convert ion soft landing into a conventional technique. Especially, research is needed to increase the efficiency of the process (total yield of soft landing) and to further investigate the nature of the interactions between gas-phase ions and the surface.

1.4 Electrospray ionization

Electrospray provides a unique MS ionization technique. The electrospray effect was first observed and reported[70] before the modern science was born. Its potential importance was first recognized early in the last century (experiments carried by Zeleny[71] between 1913 and 1918, by Chapman in the 1930s[72], and later in 1960s by Dole and Mack.[73,74] The first modern coupling with MS (ESI-MS) was, however, introduced by Yamashita, Meng and Fenn [75-77] in the 1980s. The invention of electrospray ionization was rewarded with the 2002 Nobel Prize in Chemistry, which (in part) went to John Fenn.

ESI of protic solutions produces gas-phase ions that retain all or most of the structured features of the solution molecules. With a few exceptions, electrospray ionization occurs by acid-base chemistry producing singly or multiply protonated cations (positive mode) or deprotonated anions (negative mode). Alternatively, attachment of cations (e.g. NH_4^+, Na^+ or other metal cations) or anions (e.g. Cl^-) forms gas-phase ions whose structures are related to the starting compounds in solution. Because of its flexible features, ESI has become a major technique that allows injection of large molecules into mass spectrometers.

Electrospray is by far the softest ionization technique available in mass spectrometry for macromolecules. It has been questioned if it is an ionization device or only an interface between ions in solution phase and gas phase. However, it is now recognized that there are several complex processes that all takes place during

the very last stages of ion formation and have dramatic impact on the final ESI-MS spectrum (e.g. charge competition, supercharging of ions due to the presence of additives with high surface tension, dramatic pH change and others). Thus it is reasonable to conclude that electrospray is an ionization device equivalent to other ion sources, but due to its nature it is extremely suitable for ionization of polar compounds and macromolecules.

The mechanism of ESI involves three stages: [78]

1) *Formation of small charged droplets at the tip of the electrospray capillary.* Analytes are dissolved in a polar solvent and infused through a capillary held at high potential (2-5kV, positive for positive ion mode analyses and *vice versa*) The droplets are charged because, due to the action of the applied electric field on the solution at the ES capillary tip, the meniscus of the liquid at the capillary tip becomes enriched on positive ions. Therefore, the droplets formed from the elongated meniscus (Taylor cone) contain an excess of positive electrolyte ions, when the capillary tip is positive (positive ion mode).

2) *Desolvatation.* Evaporation of solvent from the droplet decreases its radius and since the charge is conserved, at some critical radius given by the Rayleigh equation, Coulombic repulsion overcomes the surface tension of the liquid and lead to fission of the droplets. Repeated evaporation and fission lead to very small charged droplets which are the precursors of the gas-phase ions.

3) *Gas-phase ion formation.* The actual mechanism by which the gaseous ions ultimately detected with the mass spectrometer, are formed from the very small and highly charged droplets was until recently under dispute. Two models were considered, the charged residue mechanism (CRM) and the ion evaporation mechanism (IEM). Strong evidence has now accumulated that indicates that both

mechanisms apply. The IEM is involved in the production of small ions such as the conventional inorganic and organic ions, while CRM is involved in the production of macroions such as the globular proteins.

Regardless of details of its ionization mechanism, electrospray ionization has been revolutionary to the mass spectrometry and its applications.

There exist variations on the basic electrospray arrangement. The major ones are microspray (μ-spray), nanospray and even picospray[79]. The primary difference is in the reduced flow rate of the analyte containing liquid but other differences (reduced internal diameter of capillary and lack of nebulization gas) exist too. These variants are important because they generally offer better sensitivity over traditional electrospray. In addition, slower flow rate provides additional time that is needed for longer acquisition time in MS^n detection mode in case of ESI coupling with separation techniques (liquid chromatography or capillary electrophoresis).[79]

A detailed analysis of the theory of ESI-MS and its applications, including the currently accepted models for ion formation[78a,b], can be found in a recent monograph.[78c]

1.5 Surface plasma treatment

The term plasma was coined by Irving Langmuir. Plasma is made up of energetic, dissociated, positively and negatively charged particles, in addition to neutral species and therefore it is reactive towards a variety of materials. There are numerous fundamental publications[80-82] that describe the theory of plasma and its development. Plasma technology has been used to treat surfaces because one of its advantages is the versatility with which it can be applied.

Plasma treatment is particularly suitable for the specific modification of surfaces [83,84] because it can alter the surface of a the material or impart a new functionality within only the outermost layers. Therefore, the material can be made more biocompatible without changing bulk properties. Since the 1960s plasma glow discharge was applied for the treatment of tissue culture polystyrene to enhance cell

adhesion.[85] It was shown later that plasma treated surfaces display excellent cell compatibility[86,87]and low thrombogenicity. [88,89] Plasma can be used to activate the surface for covalent immobilization[90] and reactive functional group attachment. [91,92]

1.6 Computational Methods

The work in this thesis was not primarily focused on computational chemistry. Nevertheless, computational methods complement experimental results obtained by mass spectrometry and can predict the most stable gas-phase or solvated species and it basic properties. In this work computational techniques available through Gaussian 03 suite of programs [93] were used to predict vibrational spectra of soft landed species.

Density functional theory (DFT) techniques are amenable to study larger systems because DFT allows replacing the many-body electronic wave function (used by traditional methods) with the electronic density as basic quantity. DFT was originally developed by Kohn, Hohenberg, and Sham in the middle 1960's in effort to develop a method to determine the ground state energy for molecular systems solely using the electron density. DFT treats the electron cloud as a slowly changing gradient of electrons that decreases as the distance from nucleus increases.

This relies on an approximation stating that although the electron density is a gradient function, the density that any electron sees is nearly constant over a small volume.[94-99]

1.7 Project description

The experimental results presented in this doctoral thesis were obtained on a lab-built instrument that was originally designed and constructed during a collaborative project of UW researchers Buddy D. Ratner (UWEB), František Tureček (Chemistry) and W. Timothy Elam (APL). The initial work and material costs were mostly funded by Genzyme Corporation, MA.

The project was focused on new bioengineered materials and the instrument was originally meant as a surface preparation device that allows depositing of a wide scale of large biomolecular ions from the gas phase onto plasma pretreated surfaces. Expected enhancement of the surface biocompatibility was the initial goal of the project.

The instrument combines an electrospray ionization system, appropriate ion optics and rf plasma reactor. The plasma chamber is directly adjacent to the ion optics chamber. This arrangement allows treatment of the surface with plasma *in-situ* and just few seconds (or minutes) before the surface-ion collision experiment. Detailed description of this instrument is given in the Chapter 2.

Preliminary data[56] showed that this approach allows for immobilization of electrosprayed hyaluronic acid ions from the gas phase onto stainless steel surfaces and that the modified surface obtained some of the properties determined by hyaluronic coating. Nevertheless, the company finally decided not to continue with the further development. The instrument stayed available at the Department of Chemistry for the purposes of basic academic research.

My experiments were mainly focused on: determination of absolute soft landing yields; investigation of any possible structural changes that the ions could have experienced due to the surface collision; determination of bioactivity after landing; investigation of mechanism that leads to the ion discharge after the surface collision; and assessment of conditions that are needed for successful immobilization (reactive landing).

I also had to implement modifications to the original "inherited" design of the instrument.[56] These changes included construction of a movable manipulator that can provide automatic motion of the target surface, construction of electrical circuitry that allows operator to monitor the ion current on the surface, construction of flexible electrical connection between surface and the surface current monitoring system, improvement of electrospray ion source and design of a new microspray ion source.

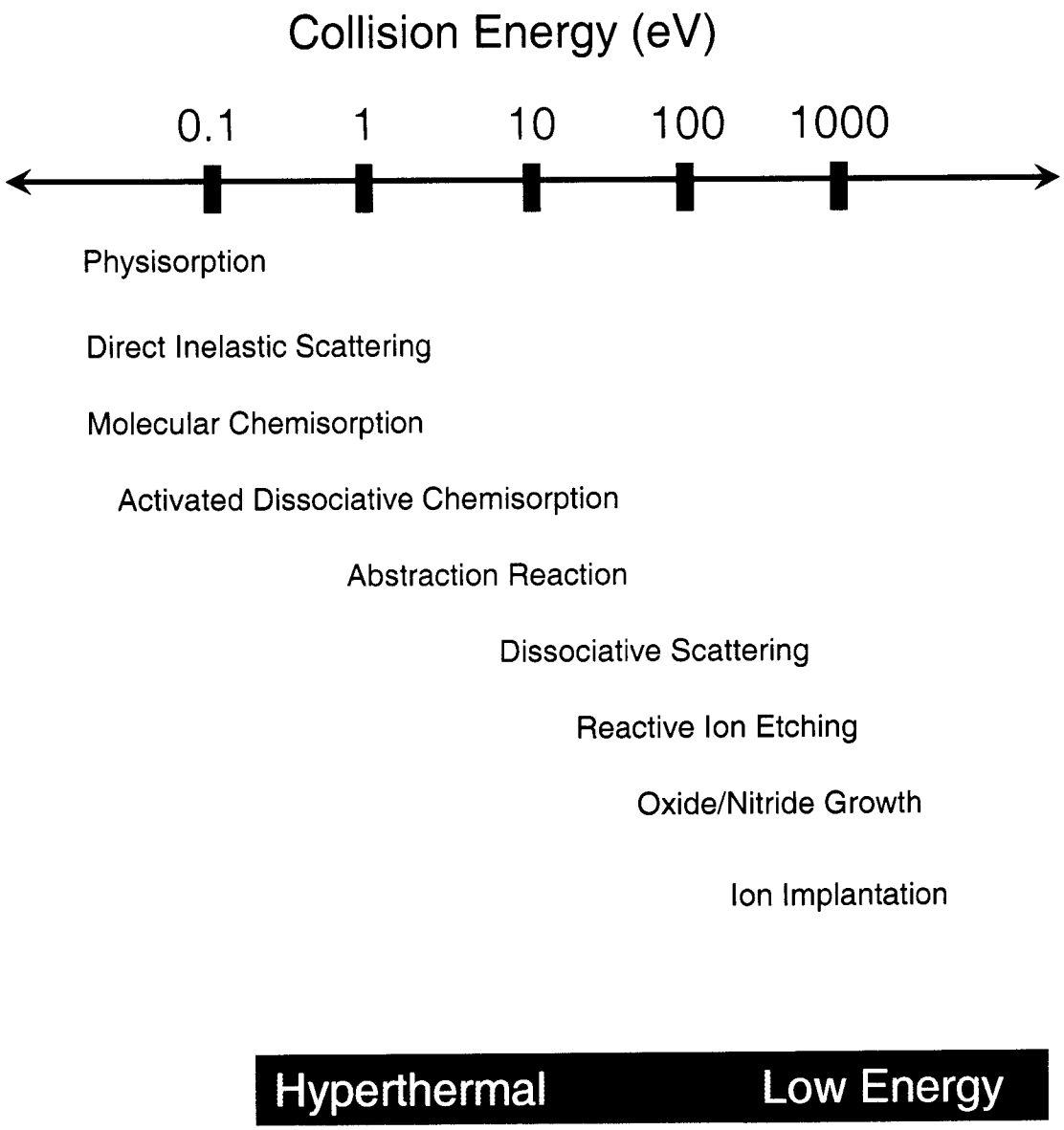

Figure 1.1 Fundamental reactive molecule/surface processes.

Each region designates the approximate ion kinetic energy range over which the associated process can be studied. Adapted from reference 24.

1.8 Notes to Chapter 1

(1) Ernest Lawrence and his group at UC Berkley demonstrated the feasibility of using modified cyclotrons for isotope separation in early 1942. The device became known as Caultron. The origin of this composite word is from "*CalU*" (University of California) and "*tron*" a Greek suffix meaning instrument.

(2) Smith, L.P.; Parkins, W.E.; Forester, A.T. *Phys. Rev.* **1947**, 72, 989-1002.

(3) Love, L.O. *Science* **1973**, **182**, 343-352.

(4) Yergey, A.L.; Yergey, A. K. *J. Am. Soc. Mass Spectrom.* **1997**, 8, 943-953.

(5) For instance permeation chromatography or some of the electrophoretic techniques. For details see any available separation techniques textbook, e.g. Robards, K. *Principles and Practice of Modern Chromatographic Techniques*, Academic Press; Elsevier, London; 1994.

(6) It needs to be stated here that presently there is absolutely no practical link between soft landing and enrichment process for the preparation of nuclear weapons. Modern techniques are superior to "Calutron like" devices in all respects. Gaseous Diffusion was introduced already in 1940s and it was used extensively in last decades. More efficient Gas Centrifuge process was developed in the 1980s (although the original idea goes back to 1934) and even introduced commercially in Germany, Great Britain and the Netherlands. Currently, the Gas Centrifuge process is being installed at the enrichment facility at Piketon, Ohio that is operated by the United States Enrichment Corporation for the Department of Energy. In addition, there are other experimental techniques in different stages of development, e.g. Atomic Vapor Laser Isotope Separation, Molecular Laser Isotope Separation and Chemical Methods (source: U.S. Nuclear Regulatory Commission, public on-line materials).

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CHAPTER 2: EXPERIMENTAL

2.1 Instrumentation

2.1.1 Soft Landing Instrument

The schema that describes the special instrument for preparative soft and reactive ion landing and implantation is shown in Figure 2.1 (Figure 2.2 shows a picture of the instrument). The instrument has two principal parts that are separated by a central gate valve: The plasma chamber (or plasma reactor) and the ion deposition chamber (or "mass spectrometric" part). The purpose of the plasma treatment is to clean the surface from residual impurities and to create an oxide layer that serves as a surface for soft and reactive landing experiments as was already mentioned in the previous chapter in the short project description.

The ion transport system uses standard electrostatic optics: Ions are generated by electrospray ionization and transported to the metal target by an electrodynamic funnel lens and an octopole ion guide. The ion motion along the electrostatic potentials is schematically pictured on Figure 2.3 and discussed in more detail in the following text.

The instrument operates as follows: A metal target is mounted on a linear translation manipulator and inserted into the plasma chamber. The custom-made plasma reactor functions like commercially available systems and is similar to those described by Ratner[1]. An rf at frequency 13.56 MHz is supplied by a 300W rf system (Dressler CESAR 133 D, Germany). The discharge can be conducted to achieve surface etching, cleaning, controlled oxide layer formation, or even a polymer film deposition. In this work two different types of plasma were used (both at the operational pressure of 250 mTorr): pure oxygen and argon/air mixture. The actual power and time used for the surface treatment were typically between 60W - 120W and 10-15 minutes, respectively. Once the plasma treatment is finished the chamber is pumped down to its base pressure (~1mtorr) and the target with the

plasma modified surface is then translated through the central gate valve to the ion deposition chamber where it can be exposed to gas-phase ions.

The electrosprayed ions are transported from atmospheric pressure to the first differentially pumped vacuum chamber by a glass-lined capillary (0.8 mm i.d.) and further by an electrodynamic funnel lens[2] that achieves efficient separation of gas-phase ions from neutral gas. The ion funnel radially focuses the ions while allowing the solvent molecules and ions of m/z ratio less than 70 to disperse and be pumped away. A dc potential gradient of 200V and an rf voltage is applied along the stacked ion funnel plates. The last funnel plate is followed with a 2 mm aperture that is biased to continue the potential gradient. The aperture represents a conductance limit for neutral molecules. The outlet side of this aperture is aligned with the axis of the octopole ion guide. The octopole operates in an rf only mode and serves to further radially focus the ions. Once the ions pass through a conductance limit and the octopole ion guide, they are intercepted by a collector plate with the landing surface that is kept as close as possible (approx. 2 mm) to the downbeam end of the octopole.

After the mass-unresolved ion beam leaves the octopole the ions are attracted by an inverse potential (bias) to the surface of a given sample (previously treated by *in-situ* plasma). The mean free path of ions in the pressure regime of the soft landing experiments (5×10^{-4} to 1×10^{-3} Torr) is estimated at >5 mm, so that the ion kinetic energy upon landing is not significantly affected by collisions with residual gas.

The inverse potential (V) on the surface determines the ion landing energy, $E_{kin} = zeV$, where z is the number of elementary charges in the ion. This value is referred to as the nominal kinetic energy because it can be moderated by surface potentials in the oxide dielectric produced by plasma etching, and in the material previously landed from the gas phase.

The octopole ion image is a circular spot of a diameter between 5-6 mm, which is the basic surface area that can be covered. Expansion of this area is possible by implementing a simultaneous motion of the target surface in the x-y plane (z being

the translation axis). In this position, the automatic motions can be switched on and the manipulator starts to move in *x-y* plane. This provides motion of the mounted target surface. The manipulator motions are controlled by a system that includes two dual-latching relays and four limiting push-button switches. The maximum surface that can be inserted in front of the octopole would be a square chip of about 2.5x2.5 cm (6.5 cm^2). However, the control system allows the usage of spacers that can effectively reduce the extent of the motion if smaller surfaces are to be covered. The relation between the original octopole spot, the limit switches set up, and the final coverage of the target is shown in Figure 2.4.

To provide bias on the surface during the motion, a flexible connection between the surface and an external power supply is necessary. This connection was solved by using a stainless steel spring with a T-shaped terminal made of gold. This terminal mates with a stainless steel hook on the manipulator and provides a flexible permanent connection with the surface. After the experiment is over, the gold terminal can be simply unhooked and the manipulator with the sample can be taken out of the instrument without any interference to the bias circuit.

The landing plate also serves as a Faraday cup detector to indicate the magnitude and stability of the ion current. In order to monitor the actual surface current – which is determined by the number of ions discharging on the surface according to the Faraday's Law – a nano-ammeter circuit (based on standard negative feedback inverting amplifier LF356) was added into the bias circuit.

Currently, the instrument is not equipped with a mass analyzer and ions regardless of mass and charge can only interact with the surface in a single channel. This should not be seen as a disadvantage because the process of interaction of ions with the surface (soft or reactive landing) is independent of the process of ion separation and depends only on the surface properties and ion energies and structures. That is why the results obtained here are directly applicable to multichannel mass spectrometric systems.

A drawback of including a mass analyzer is the unavoidable decrease of ion transmission because the presence of a mass filter lowers the number of ions that can go through the ion optics system. Data obtained on a different instrument in the Turecek group – that is equipped with an ion source of the same design – show that mass separation into 16 channels resulted in a decrease of soft landing yield by a factor of approximately ten.

For reactive landing studies that are focused on the surface-bound molecules and only require depositing high femtomole to low picomole amounts of material, a ten-fold decrease of ion current would not be critical. The current soft landing instrument can routinely achieve 1-10 nanoampere ion currents at the target. This corresponds to a mass flux of 10-100 fmol/s, or 36-360 pmol/h for singly charged ions. Even at a 10% ion transmission through the mass filter, it would be possible to deposit a monolayer of protein in a few hours of soft landing. Recently published data indicate that transmissions as high as 22% can be achieved on quadrupole instruments,[3a] which is in line with data obtained on Extrel quadrupole mass filters by Turecek.[3b]

2.1.2 Conventional instrumentation

2.1.2.1 Mass Spectrometry

Electrospray mass spectra (ESI-MS) were recorded using a Bruker Esquire quadrupole ion trap instrument equipped with an orthogonal electrospray interface. The basic electrospray conditions were as follows: ion polarity positive, drying gas flow rate 3L/min, drying gas temperature 250-300°C, ESI voltage 4kV.

2.1.2.2 Spectroscopy

XPS spectra were taken on a Surface Science Instruments (SSI) S-Probe ESCA instrument. This instrument uses a monochromatized Al Kα X-ray source for photoemission stimulation and a low energy electron flood gun for charge

neutralization. The Service Physics ESCAVB Graphics Viewer program was used to determine peak areas.

UV/visible absorption analyses were carried out on an HP8452A Diode Array Spectrophotometer (Hewlett-Packard, USA).

Fluorescence analyses in solution were carried out on LS-50B Fluorescence/Luminescence spectrophotometer (Perkin Elmer, Boston, Massachusetts, USA) with a pulsed high pressure xenon source. The excitation and emission slits were both set to 5 nm, and the scan speed was typically 50 nm/min. The excitation and emission wavelengths used for determination of actual compounds are given in the text.

Fluorescence analyses of surfaces were performed on a Molecular Dynamics FluorImager SI fluorescence scanner (now Amersham Biosciences Corp, Piscataway, NJ, USA) equipped with a 488 nm argon-ion laser excitation source. The fluorescence scans were analyzed and exported using ImageQuantNT software from the same company.

2.1.2.2 Scanning Microscopy

Atomic force microscopy (AFM) measurements were taken in air at room temperature in tapping mode using a Digital Multimode IIIa (Digital Instruments, Santa Barbara, USA). The data were analyzed by the Digital Nanoscope software version 5.12r3 from the same company.

Scanning electron microscope (SEM) images were taken using a Sirion XL-30 scanning electron microscope (FEI Company Hillsboro, Oregon, USA). Prior to the SEM analyses the samples with landed protein were coated by a 5 nm layer of gold to avoid charge accumulation due to insufficient surface conductivity. The same gold coated samples were used for AFM measurements.

2.1.2.3 Confocal Raman Microscopy

After thoroughly rinsing with methanol and water, the soft-landed samples were placed in an enclosed confocal microscope sample chamber[4] and illuminated with the 514.5 nm line from an Ar ion laser (Coherent Innova 300) that was focused to a 0.8 μm^2 spot. The scattered light from the sample was collected by the objective lens, redirected, by a series of optical elements, through the confocal microscope pinhole, and focused on the slit of a single-pass Acton SpectraPro-500i monochromator. A Kaiser Optical Systems Supernotch-Plus holographic filter was placed in front of the spectrometer to remove the laser line. The spectrum was recorded using a Princeton Instruments, back-iluminated, liquid nitrogen-cooled 1340 x 1000 pixel array charge coupled device (CCD) camera.[5] The laser beam power was typically between 100-250 mW and the exposure time was 60, 300 or 600 seconds. The spectra were smoothed by Adjacent-Averaging (AA) algorithm with 13 points considered for smoothing.

The spectra of soft-landed material were reproduced on a commercial Renishaw inVia Raman microscope using 514 nm laser excitation and a monochromator grating density of 1200 lines/mm. The wavenumber scale was calibrated with the Raman band of a reference Si (111) surface at 520 cm^{-1}.

2.1.3 Calculations

Standard ab initio and density functional theory calculations were performed using the Gaussian 03 suite of programs.[6] Structures were fully optimized with density functional theory calculations using the B3LYP functional[7] and the 6-31+G(d,p) basis set and characterized as local minima by harmonic frequency calculations including Raman intensities. Optimized geometries and Raman spectra of aqueous molecules and ions were calculated using the refined Polarizable Continuum Model[8] and B3LYP/6-31+G(d,p). Improved energies were obtained by single point B3LYP and Møller-Plesset (frozen core) calculations with the 6-

311+G(2d,p) basis set. The B3LYP and MP2 energies were averaged in the B3-MP2 scheme[9] and used to provide improved relative energies.

2. 2 Materials and Procedures

2.2.1 Chemicals

Unless stated otherwise, all inorganic compounds were purchased from Sigma-Aldrich and used as received. Solvents and buffers were purchased from Fisher and were of HPLC grade or certified purity. Methanol (Fisher, HPLC grade) was redistilled and stored in pre-boiled Pyrex containers to remove dissolved alkali metal ions.

Trypsin (11500 BAEE units/mg) and biotin were purchased from Sigma-Aldrich. Streptavidin, streptavidin AlexaFluor-488 conjugate and streptavidin OregonGreen-488 conjugate were purchased from Molecular Probes and used as received. Crystal violet, rhodamine-B, cytidine, lysine, Phe-Leu, Phe-Val, N,N-dimethylformamide (DMF), zirconium(IV) propoxide and diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich. Val-Leu-Lys-*p*-nitroaniline and Val-Val-Lys were purchased from Bachem and ^{15}N-lysine from Isotec USA. Streptavidin immobilized on agarose beads CL-48 was purchased from Fluka. 4-fluoro-7-nitrobenzofurazan (NBD-F) was purchased from AnaSpec, San Jose, CA, USA. The activated biotin derivate, biotinyl-3,6,9-trioxaundecanediamine, was purchased from Pierce.

The biotin-NBD conjugate was synthesized according to the following procedure: To a round-bottom flask containing a solution of 20 mg of biotinyl-3,6,9-trioxaundecanediamine in 0.5 mL of dry redistilled DMF and 0.5 mL of DIPEA at 0°C was slowly added a solution of 9.7 mg NBD-F in 0.15 mL of dry DMF. After stirring for 8 h at 0°C the solvent was removed *in vacuo*. The product was purified on a silica column packed with 8:2 dichloromethane:methanol. The column was eluted with the same solvent mixture to obtain 26 mg (93 %) of product. ESI-MS (M

$+ H)^+$: 538.2, $(M + Na)^+$: 560.4. UV/visible (CH_3OH): λ_{max} = 462 nm, Fluorescence (CH_3OH): λ_{max} = 539 nm (excitation at 462 nm).

The hyaluronan anions were obtained by electrospraying solution of 420 kDa sodium hyaluronate salt (Genzyme Corporation, MA Lot#9149-10A3) at a concentration of 100 nM in 85/15 methanol/water. The human blood upper physiological values for washing solutions (sodium chloride and urea) were obtained from reference[10] and both solutions were prepared using HPLC grade water. The serum was obtained from UWEB, Seattle, WA under the label FBC CB 7/1/03.

The chemical structures and origins of samples obtained from Combimatrix Corporation are subject of ongoing project and possible patent protection and can not be discussed here.

2.2.2 Landing Surfaces

All stainless steel sample surfaces were cut as approximately 20x20 mm chips. Used material was type 316L, which is a low carbon, high quality, stainless steel (Fe, <0.03% C, 16-18.5% Cr, 10-14% Ni, 2-3% Mo, <2% Mn, <1% Si, <0.045% P, <0.03% S) specially used for different medical devices. After cutting, the chips were mechanically polished with diamond paste, rinsed with pentane, methanol and water, sonicated in a 1:1 chloroform/methanol mixture for 15 minutes, rinsed again with methanol, dried and exposed to plasma treatment immediately after cleaning.

Silver foil (Sigma-Aldrich, 99.9%, thickness 0.1 mm,) was cut into approximately 15 × 15 mm chips. Prior to plasma treatment the chips were washed thoroughly with water/methanol/propanol, sonicated in hexane, rinsed with methanol, and allowed to dry.

2.2.3 Adhesion tests from platelet rich plasma (PRP)

Whole blood was obtained from normal donors and collected into acid citrate dextrose. Citrated blood was centrifuged at 800 rpm for 20 min at room temperature. PRP was removed from the supernatant above the residual red and white blood cells. PRP (200–250 mL) of approximately 300 000 platelets/mL was added to the center of each of the surfaces undergoing test for platelet adhesion and incubated for 20 min at room temperature. The samples were then rinsed with phosphate buffered saline, fixed with 3.7% formaldehyde for 15 min, and rinsed with phosphate buffered saline. The fixed samples were stained for 90 min with 1% Coomassie Blue, destained with 5% acetic acid, and submitted to scanning electron microscopy (SEM) [11]. It should be noted here that the cell adhesion tests presented in this work were done by Genzyme Corporation, MA in their facilities and by their researchers.

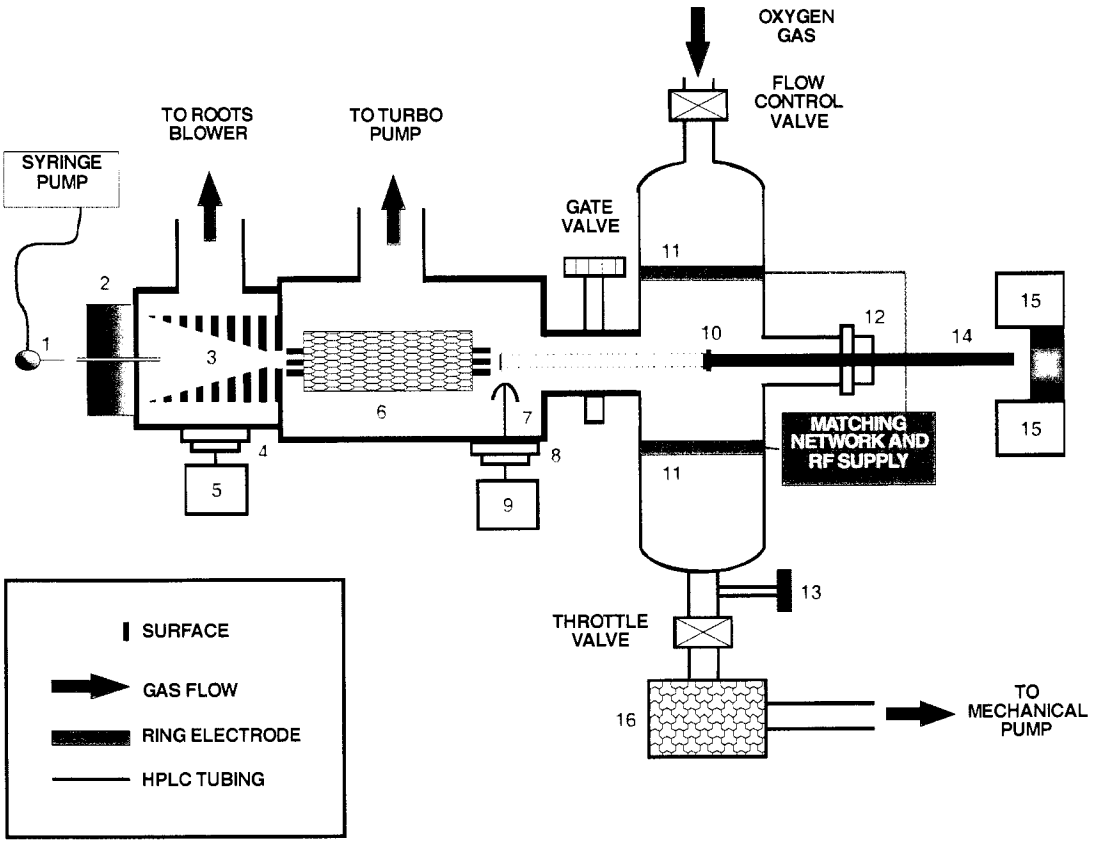

Figure 2.1 Schematic diagram of the apparatus for soft and reactive landing.

Drawing is not on scale and vacuum flanges are not shown. **1**: Microspray ion source with the PicoTip emitter. Inset shows a photograph of the microspray setup. **2**: Heated counter electrode plate with the inlet capillary. **3**: Ion funnel. **4**: DC and RF power feedthroughs for the ion funnel. **5**: DC and RF power supplies for the ion funnel. **6**: Octopole ion guide. **7**: Flexible fishhook line for electrical connection to the soft-landing plate. **8:** Electrical feedthroughs for the octopole and soft-landing plate. **9:** DC and RF power supplies for the octopole and a nanoamperemeter for ion current monitoring on the surface. **10**: Soft landing surface (the dotted lines indicate the movement of the linear motion manipulator from the plasma reactor into the soft landing chamber). **11**: Cu ring electrodes on the Pyrex plasma reactor. **12**: Linear motion feedthrough. **13**: Overpressure safety valve (burst disk). **14**: Sample surface manipulator and holder. **15**: Power and control system for manipulator motion in the *x*-*y* plane (*z* being the translation coordinate). **16**: Molecular sieve and cryo-trap for the vacuum line to the plasma reactor.

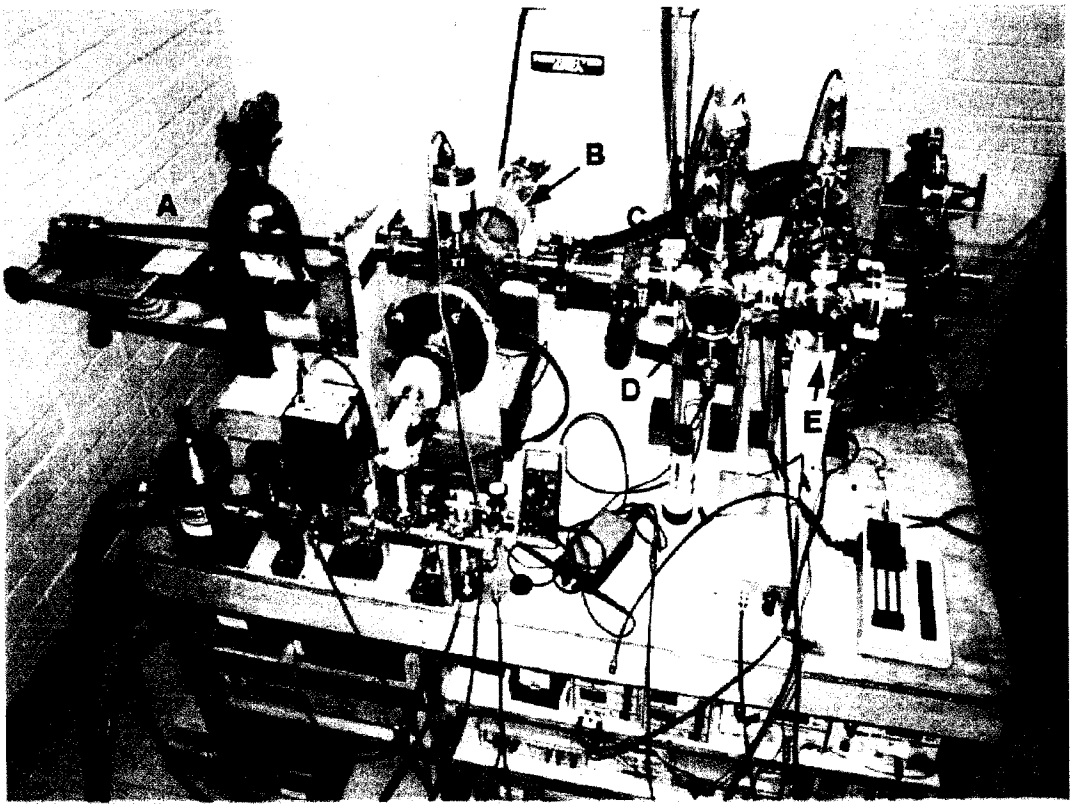

Figure 2.2 Overall view of the apparatus for soft and reactive landing.

A: Sample manipulator, B: plasma chamber, C: gate valve, D: high vacuum region for soft landing, E: low vacuum region with the funnel lens, F: electrospray ionizer in a Plexiglass high-voltage protection box. The ports on the top of the instrument carry vacuum gauges.

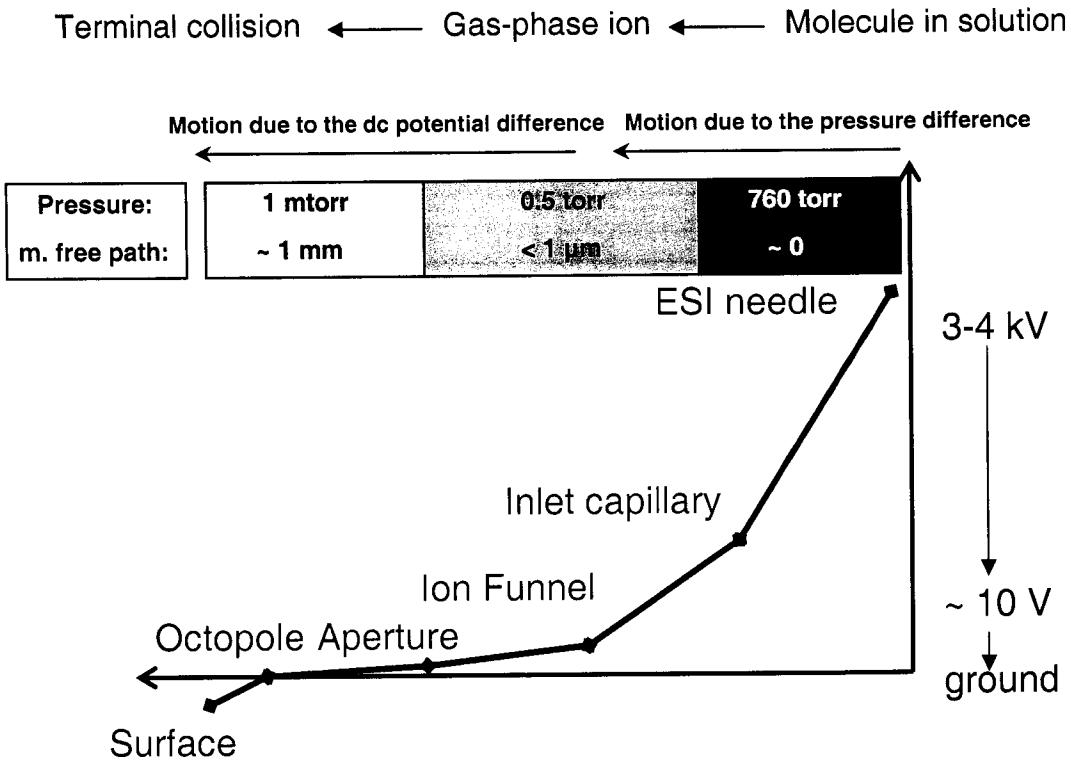

Figure 2.3 Ion motion and electrostatic potentials inside the instrument.

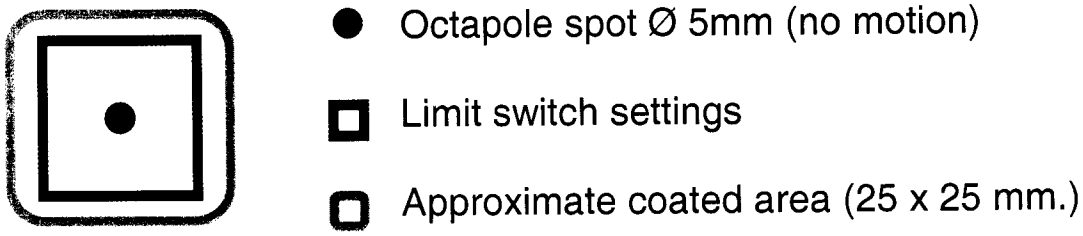

Figure 2.4 The relation between coated area and limiting switches.

2.3 Notes to Chapter 2

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CHAPTER 3: PREPARATIVE SOFT AND REACTIVE LANDING OF MULTIPLY CHARGED PROTEIN IONS

3.1 Introduction

The concept of ion soft landing was first proposed by Cooks[1] without an immediate application purpose. Currently, one of the strongest motivations for development of soft/reactive landing techniques is the increasing interest in protein microarrays and other sensor devices that use specific interactions with immobilized proteins to detect analytes. There are a several reviews that describe the current state of protein arrays technology and applications currently available.[2]

Nondestructive landing of proteins from the gas phase seems to be very promising technique for protein arrays preparation[3] because the position of landed molecule - as well as the shape of the deposited nanostructure - can be directly controlled by electrostatic ion optics that guides ionized molecules inside the mass spectrometer.

In addition, one of the problematic issues of current protein arrays is nonspecific sorption of molecules - that should be specifically detected via the interaction with protein microarray - on currently used soft surfaces (for instance polyacryalamide, nitrocellulose or agarose). Reactive landing is both more universal and capable of depositing proteins on surfaces that have lower tendencies to support the interfering nonspecific interactions.

The question is, however, if the protein structure and biological activity can survive landing on dry metal surface. That is why the key experiments in this work were focused on reactive landing of proteins.

3.2 Soft and Reactive Landing of Multiply Charged Trypsin Ions

Enzymatic activity represents a sensitive probe of structural integrity of large molecules. Cooks and co-workers have shown that soft landing of multiply

protonated protein ions into liquid droplets resulted in retention of biological activity.[3]

Trypsin and other enzymes have been shown to retain their enzymatic activity after their multiply-charged gas phase ions were soft-landed into liquid droplets.[3,4] Trypsin is a protease that is widely used for residue-specific protein degradation.[5] Because of its rugged structure that is enforced by six disulfide bridges[6], trypsin was deemed to be a suitable enzyme model for soft landing studies on plasma treated metal surfaces.

ESI of bovine trypsin produces a distribution of multiply protonated gas-phase ion species, $(M + nH)^{n+}$, n = 10-15, which peaks at the $(M + 13H)^{13+}$ ion of m/z 1793 (Figure 3.1). Although in this instrument the individual charge states produced by ESI are not separated, previous results indicated that they behave similarly upon soft landing[3b]. ESI on the soft-landing instrument of 10 μM bovine trypsin in 8:2 methanol-water generated 1-2 nA ion currents as measured on the metal target surface. Seven sample surfaces were prepared at landing potentials 0, 5, 7, 10, 12, 15, and 20 V, and the soft-landing experiments at all these potentials were reproduced over the period of two months. The respective charge-averaged nominal landing energies ranged from near-thermal to 65, 91, 130, 156, 195, and 260 eV.

The activity of the soft-landed and immobilized trypsin was monitored by a surface-compatible solution assay that was based on cleavage of a tripeptide conjugate, VLK-p-nitroaniline, and product detection on a Bruker Esquire Quadrupole Ion Trap with tripeptide VVK as an internal standard. The quantification of the landed enzymatic activity was based on the comparison with the calibrated activity of free trypsin in solution.

Each sample surface with landed enzyme (or blank surface for control experiments) was positioned on the flat bottom of a large plastic vial. 2 mL of 10^{-4} M substrate solution (VLK-p-nitroaniline) in 50mM NH_4HCO_3 water solution (pH 7.8) and 100μL of deionised water were added into the vial. Calibration standards were treated exactly the same way but 100μL of trypsin water solution of known

concentration was added instead of pure deionized water. The vials were closed tightly and kept in a water bath. The assay was allowed to react for seven hours at 37 °C. After that the vials were removed and quickly cooled on ice. 100 μL of assay solution was then taken into 1mL of 9:1 methanol/water and 20μL of $5.8*10^{-5}$ M solution of internal standard (VVK) were added into each sample. All samples were then analysed by ESI-MS.

Sample surfaces were always removed from assay mixture rinsed with water/methanol and dried to avoid contamination from the previous run. After that they were ready for the next assay run.

Four consecutive assays were run with each sample surface, and the decrease of activity after each run was monitored until only a residual activity was obtained. It is expected that during the first assay, all soft-landed trypsin is washed into the substrate solution. The detected activity of this soft-landed trypsin did not critically depend on the potential applied to the metal plate, but on the ion current only. After quantification, the amount of trypsin determined by the assay was compared with the amount calculated according to Faraday's law from the known ion current, deposition time, and number of charges. The obtained fractions of active enzyme varied for each surface between 72 and 98% and were reproduced within this range for another set of soft-landed samples prepared two months later. The calculated average fraction of trypsin molecules that survived the collision without loss of enzymatic activity was 84%. The results of the second, third, and fourth consecutive assays are shown in Figure 3.2. Remarkably, even though most of the enzyme has been washed in the first assay, the surface still retained substantial trypsin activity. This activity could only be attributed to the trypsin molecules that were chemically bound to the active layer on the surface.

The properties of the soft-landed layer on the stainless steel surface were investigated by X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), and atomic force microscopy (AFM). Table 3.1 shows results of XPS analyses of the outermost 2-10 nm of the trypsin layer that was soft-landed on

stainless steel and compares it with XPS of a blank, plasma- treated stainless steel surface. There is a significant decrease of the iron and chromium content on the surface that was exposed to trypsin ions. Conversely, the exposed surface shows an increased content of nitrogen indicating that a protein material was deposited there.

Figure 3.3 compares SEM and AFM scans of surfaces with (Figure 3.3 a,d) soft-landed trypsin, (Figure 3.3 b,e) reactively landed trypsin after extensive washing by four solution assays, and (Figure 3.3 c,f) a blank plasma-treated surface. Both SEM and AFM of soft-landed trypsin show contours of structures that are formed by large trypsin domains. SEM does not provide a good distinction between the blank and exposed and washed sample surfaces, but AFM clearly shows that the exposed surface is covered by at least one uneven layer of trypsin molecules even after the soft-landed enzyme had been washed off. These experiments show that deposition of multiply-charged trypsin ions on a dry hard surface that was in-situ pretreated with oxygen plasma resulted in the formation of several layers of material.

The soft-landed trypsin, which represents the upper layer, can be washed from the surface into solution with only minor loss of activity. Because the activity virtually does not depend on the landing potential even for highly hyperthermal ions, it is reasonable to conclude that the inner layers of trypsin that are deposited first form a protective coating that makes the surface behave analogously to the liquid droplets reported previously.[3,4]

The difference between the AFM and SEM scans before and after the washings, as well as the results of consecutive assays, confirm that after the upper layers of the soft landed enzyme have been washed off into the solution, there is still active trypsin that is bonded to the surface. The data indicate that the activity of the bound trypsin is lower than that of the soft-landed enzyme. We estimate that, considering a 100 Å diameter for the trypsin molecule, the spot of 0.28 cm^2 area covered by the enzyme can contain 2.8 pmol of trypsin per monolayer. Figure 3.2 shows that the residual activity of samples landed at 12 and 15 V corresponds to an equivalent of 1.5 pmol of trypsin. This indicates that about one half of the trypsin

molecules in a closely packed monolayer remain active. The unexpectedly large fraction of active surface-bound trypsin is most likely due to its compact structure that is stabilized by six disulfide bonds.[6]

The resolution of the AFM scans (Figure 3.3) does not allow one to establish the size and packing of the individual trypsin molecules in the surface-bound layer. Previous work by Reimann et al. of multiply charged protein ions impinging on the surface at 146 keV showed individual surface defects (hillocks) due to protein ion impact that had diameters as large as ~20 nm. [7]

3.3 Soft and Reactive Landing of Multiply Charged Streptavidin and AlexaFluor-488 Labeled Streptavidin Ions.

Streptavidin is a homotetrameric protein that is widely used for bioaffinity studies.[8] Electrospray ionization of streptavidin produces three major series of $(M+nH)^{n+}$ ions, n = 7-11. Two of them peak at the $(M + 7H)^{7+}$ ion of m/z 1854 (series A) and m/z 1864 (series B) and the third peaks at the $(M + 9H)^{9+}$ ion of m/z 1483 (series C) in an A:B:C ≈ 2:1:0.75 ratio of intensities (Figure 3.4). Deconvolution of all series gave the corresponding molecular masses as 12972 Da, 13042 Da and 13336 Da for A, B and C, respectively, that match the known sequence of the streptavidin precursor, e.g., residues 10-135 for series A, residues 37-160 for series B, and residues 19-147 for series C (14). The mass spectra indicate that the streptavidin tetramer dissociates to monomer units upon ESI. Electrospray of Alexa Fluor-488 labeled streptavidin (M_r= ~53 kDa) also showed a series of $(M + nH)^{n+}$ ions, n = 7-9, corresponding to a monomeric protein. The electrospray ionization of streptavidin was previously reported by Schwartz et al. who found both tetrameric and monomeric ions depending on the ion source conditions.[9] For preparative soft-landing, Alexa Fluor-488 labeled streptavidin was electrosprayed from a 1.9×10^{-6} M (1 mg/10 mL) solution in 3:2 methanol:water and the ions were deposited on a freshly plasma treated stainless steel surface that was biased at -20 V. This corresponds to nominal landing energies in the range of 120-160 eV for the major

multiply charged ions. The deposition lasted for 4 h and 1 mL of solution was electrosprayed. The soft-landed material was recovered from the surface by rinsing with 1 mL of 3:2 methanol:water and analyzed by fluorescence spectrophotometry according to a calibration curve at 528 nm (excitation at 488 nm). The total determined amount of Alexa Fluor-488 streptavidin was 0.3 μg (5.7×10^{-12} mol), which corresponded to a 0.3% overall solution-to-solution yield. The yield indicates that the amount of recovered material per one hour of deposition was similar to that obtained for trypsin.

It is worth noting that after approximately three to four hours of streptavidin or AlexaFluor-488-streptavidin landing, the ion current that was measured on the metal target dropped to close to zero. When the electrospray was stopped, the soft-landing experiment was paused for approximately 15-30 minutes and then restarted, the surface current rebounded to the initial value. This indicates that a thick layer of streptavidin already deposited on the surface hinders ion discharge, which becomes the rate-limiting step for further ion deposition. Interestingly, we did not observe the same phenomenon when soft-landing other large multiply charged ions, e.g., those from trypsin and sodium hyaluronate. [10,11]

Another surface was exposed to approximately the same amount of Alexa Fluor-488 streptavidin, based on the deposition time and surface current, by soft landing at 120-160 eV. The surface was scanned for fluorescence immediately after the landing experiment (Figure 3.5). After the first scanning, the surface was washed by 100 μL of PBS (15 min) and the recovered solution was stored for quantification. Then the surface was washed successively by 100 mL of PBS (2 h), 100 mL of PBS (2 h), 100 mL of PBS (2 h), 100 mL of 25 mM sodium hexyl sulfate in PBS (1 h) and 100 mL of 25 mM sodium hexyl sulfate in PBS (1 h). All solutions were stirred during washing to ensure proper mixing. Surface fluorescence was monitored after each washing step, and the average and total pixel brightness of the original landing spot area were recorded. Figure 3.6 shows a plot of the relative surface fluorescence (total pixel brightness) in the process of target washing. During the first two hours of

washing more than one half of the soft-landed material was stripped by the buffer solution. The washing was particularly efficient during the first 15 minutes of exposure to solvent. This demonstrates that a substantial part of soft-landed streptavidin can be reconstituted into a buffer solution in a matter of minutes, which may be practically important for preparative isolation. After 2 hours, further washings with PBS buffer caused only a negligible decrease of fluorescence of the surface-bound material. Washing with sodium hexyl sulfate resulted in a further decrease of fluorescence. This decrease could be caused by removal of streptavidin from the surface or its denaturation, which could affect fluorescence, as well. Interestingly, the streptavidin-coated surface retained above-background fluorescence even after prolonged washing with the detergent (Figure 3.6).

Another fluorescent labeled streptavidin derivative was used to investigate if non-specific adsorption of protein occurred on plasma-treated stainless steel surfaces. In this experiment, a freshly plasma-treated surface was exposed to a solution of 1 mg of OregonGreen-488 streptavidin conjugate in 3 mL of PBS buffer. After 1 hour the surface was rinsed with water and scanned for fluorescence. However, the measured fluorescence intensity was the same as that obtained from a blank surface (Figure 3.5). Hence, no non-specific streptavidin adsorption was detected. Possibly the surface quenched a signal associated with low levels of adsorption. Further experiments were carried out to investigate whether soft-landing affected the protein structure.

Streptavidin (2:1:0.75 mixture of A, B, and C forms) was electrosprayed from a 1.9×10^{-6}M (1 mg/10 mL) solution in 3:2 methanol:water and landed on a freshly plasma-treated stainless steel surface at 120-160 eV. The surface was washed into 300 μL of water, 300 μL of acetonitrile was added, and the solution was analyzed by ESI-MS. The mass spectrum of soft-landed and reconstituted streptavidin was practically identical to that of the original sample in that both contained the same series of monomeric protein ions with similar charge distributions and produced the same deconvoluted molecular masses for series A, B,

and C ions. Likewise, no changes were observed for bioaffinity to biotin of soft-landed streptavidin, as tested with AlexaFluor-488-labeled streptavidin. The soft landed protein was washed from the surface with methanol, the fluorescence of recovered AlexaFluor-488-streptavidin was measured, and an excess of biotin (0.41 μmol) was added to the solution. The same procedure was carried out with the calibration solution of AlexaFluor-488-streptavidin. Both solutions showed at least 5 fold decrease of fluorescence intensity as a result of biotin binding. Although this effect is undoubtedly due to biotin conjugation with the protein, it should be noted that opposite effects, e.g., fluorescence enhancement upon biotin attachment, have been reported for other fluorescent labels, such as BODIPY[12].

The surface with reactively landed streptavidin that had been thoroughly washed was exposed to a 3.1 mM solution of a fluorescent-labeled biotin-NBD conjugate (Bio-NBD, Figure 3.7) in 50:50 methanol:water for three hours. The surface was then removed, repeatedly washed and rinsed with methanol, and scanned for fluorescence. However, no fluorescence that could be attributed to a surface-bound Bio-NBD was detected. The same surface was then treated with a saturated aqueous solution of biotin for 3 days and the wash solution was analyzed by fluorescence spectroscopy. The spectrum obtained (Figure 3.8) clearly shows the emission band of Bio-NBD at 539 nm compared to the blanks from a biotin solution and a wash biotin solution from another plasma-treated metal surface that was previously exposed to the solution of same Bio-NBD conjugate and washed with methanol. The amount of Bio-NBD displaced from the surface was determined from a calibration curve as 9 pmol. Because of the 1:1 stoichiometry of biotin-streptavidin conjugation, 9 pmol represents the lower bound of the streptavidin monomer immobilized by reactive landing on the metal surface.

3.4 Discussion

Soft landing on plasma-treated metal surfaces produces functioning proteins in preparatively significant yields. However, differences apparently exist between the

major protein fraction that is washable to solution and the fraction in the underlying layer that is immobilized on the metal oxide surface. In the case of trypsin, electrospray ionization forming multiply protonated ions evidently does not cause protein unfolding and denaturation, which applies to both soft and reactively landed ions. The first monolayer of trypsin cations is thought to land with the nominal kinetic energy and discharge by proton transfer to the metal oxide layer created on the surface of bulk stainless steel by plasma oxidation. Note that multiply charged gas-phase ions are acids whose reactivity for proton transfer is limited from above by the basicity of the solvent used in electrospray. Considering the low gas-phase basicity of methanol (728 kJ/mol), multiply protonated protein ions can be considered strong acids that should be able to transfer protons onto amphoteric metal oxides such as Fe_2O_3. The metal oxide layer is thought to mediate charge transfer whereby Fe^{3+} cations in the protonated oxide layer are reduced to Fe^{2+} by electron transfer from the bulk metal conductivity band.[13] The metal oxide layer protects the multiply-charged protein ions from discharging by direct electron transfer, which would result in radical formation and dissociation.[14] Once neutralized by deprotonation, the soft-landed protein molecules can work as a base for the next layer of ions arriving from the gas phase and relay proton migration to the metal oxide layer for charge neutralization. The finding that about one half of surface-immobilized trypsin molecules are catalytically inactive can be due to chemical damage to the binding cavity or amino acid residues that are involved in proteolysis. Since the trypsin molecules on the surface probably cannot reorient [15], the decrease of catalytic activity may also be due to a fraction in which the binding site is oriented toward the metal surface and thus is made inaccessible to the substrate.

The base can also mediate energy transfer from the impacting gas-phase protein ions that land at hyperthermal kinetic energies. The range of actual landing energies, as opposed to the nominal zeV values (see section 2.1.1 for more details) that represent an upper bound, is unknown at present. For example, the presence of protonated molecules in the underlying protein layer can create local space charge

that would affect the electrostatic potential in the vicinity of the soft-landing ion. The magnitude and distribution of space charge effects are unknown at present, although the soft-landing yields indicate that space charge effects do not prevent the gas-phase trypsin ions from reaching the surface. The fact that the yield and biological activity of the upper layers of soft-landed trypsin do not strongly depend on the electrostatic potential applied to the collector metal plate may indicate the presence of an effective potential $E_{eff} < zeV$ on the surface.

The results obtained for streptavidin and its fluorescent-labeled derivatives combine effects of gas-phase ion structure and surface interactions. Streptavidin, which forms a thermally stable tetramer in solution [16], is found to partially or fully dissociate to multiply charged monomer units upon electrospray in our experiments. According to Schwartz et al [9], the dissociation depends on the ESI interface temperature and can be expected to be thermally driven on the time scale of droplet formation and drying.[17] Monomeric streptavidin ions in the upper layers of the soft-landed material that are discharged by proton transfer on the surface and washed into solution can reconstitute dimers and tetramers which have the highest binding affinity for biotin due to cooperative binding. [18] In contrast, monomeric streptavidin in the layers that are immobilized on the surface is unlikely to form tetramers and thus may have a lower affinity to biotin. These effects are well known in solution where monomeric forms of avidin have binding constants several orders smaller than those for the tetrameric form. [19] In spite of immobilization, a substantial fraction of surface-bound streptavidin shows specific affinity to Bio-NBD. From the projected area of the streptavidin monomer molecule (625 $Å^2$) [20] and the soft-landed spot area (0.28 cm^2), it can be estimated that about 7.5 pmol of streptavidin fit within a closely packed monolayer. Thus, the 9 pmol of bound Bio-NBD points to at least 2 monolayers of surface-bound streptavidin. It should be noted that surface-bound streptavidin can lose its biotin-binding affinity by various mechanisms, e.g., by unfavorable protein orientation on the surface that can block access to the binding pocket, or by partial unfolding or damage in the amino acid residues in the binding

pocket or the flexible loop. [21] If two monolayers of soft-landed streptavidin are surface-bound, the fraction of active protein (60%) is comparable to the fraction of active surface-bound trypsin (53%).

3.5 Conclusion

Soft landing of multiply charged trypsin and streptavidin ions on dry plasma-treated metal surfaces results in a substantial recovery of functional protein. The soft-landed protein is found to form two layers on the surface. The top thick layer, which contains a major fraction of the soft-landed material, is loosely bound and can be washed in solution without damage and loss of protein function. The bottom layer, which is estimated to be 1-2 monolayers thick, remains tethered to the metal oxide surface and shows 50-60% biological activity that does not appear to critically depend on the protein structure. Both the conformationally rigid trypsin molecule and the flexible streptavidin monomer retain substantial protein activity in the immobilized form.

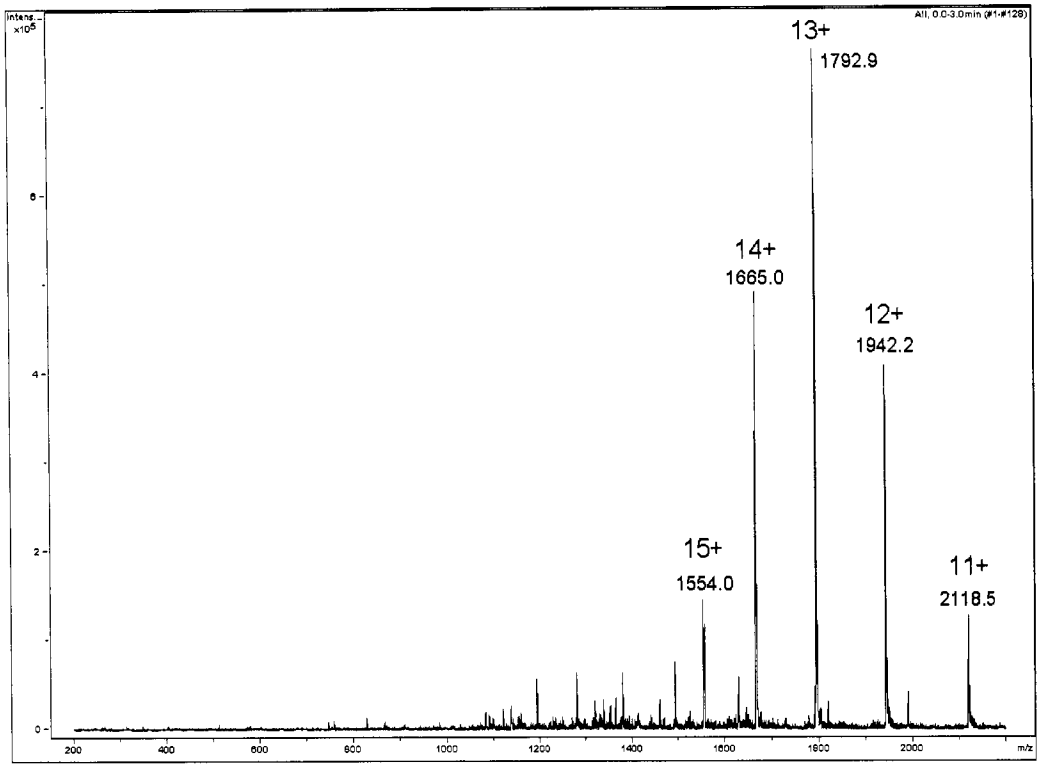

Figure 3.1 Electrospray mass spectrum of bovine trypsin.

Spectrum was obtained on a Bruker Esquire quadrupole ion trap mass spectrometer with electrospray ionization. It shows charge distribution characteristic for ESI-MS of proteins.

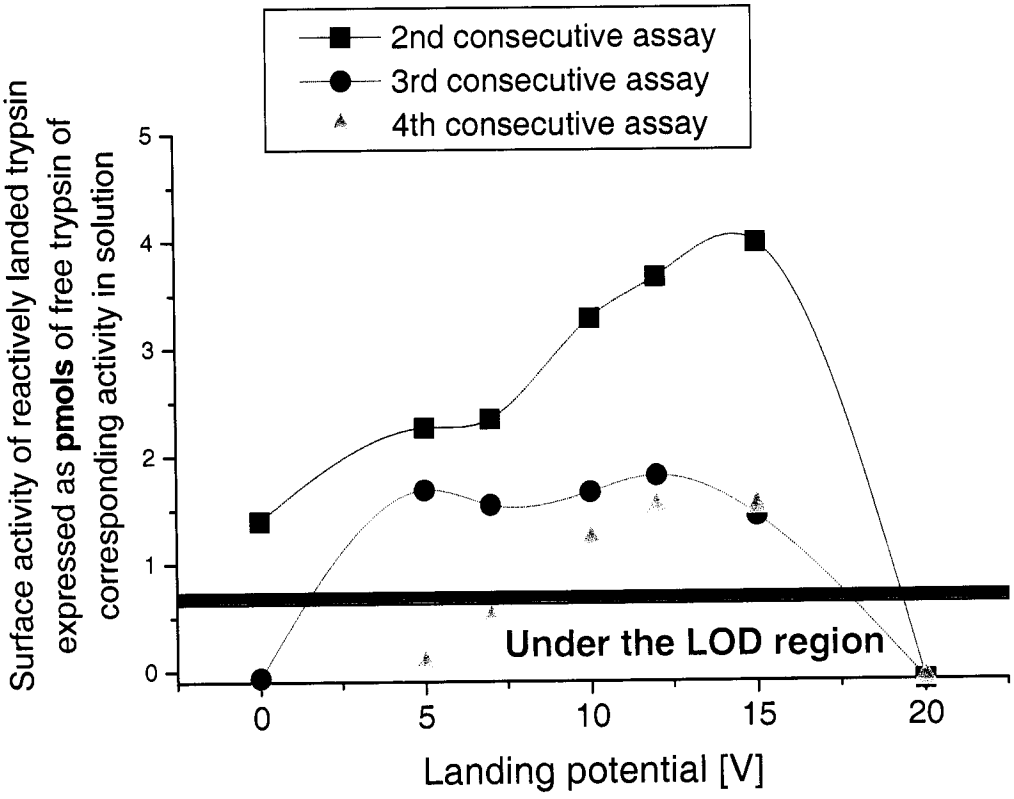

Figure 3.2 Trypsin activity assay.

Trypsin activities as measured by dipping the metal target with the deposited enzyme in a solution assay at 37 °C. Activity is expressed as picomoles of equivalent free trypsin in solution.

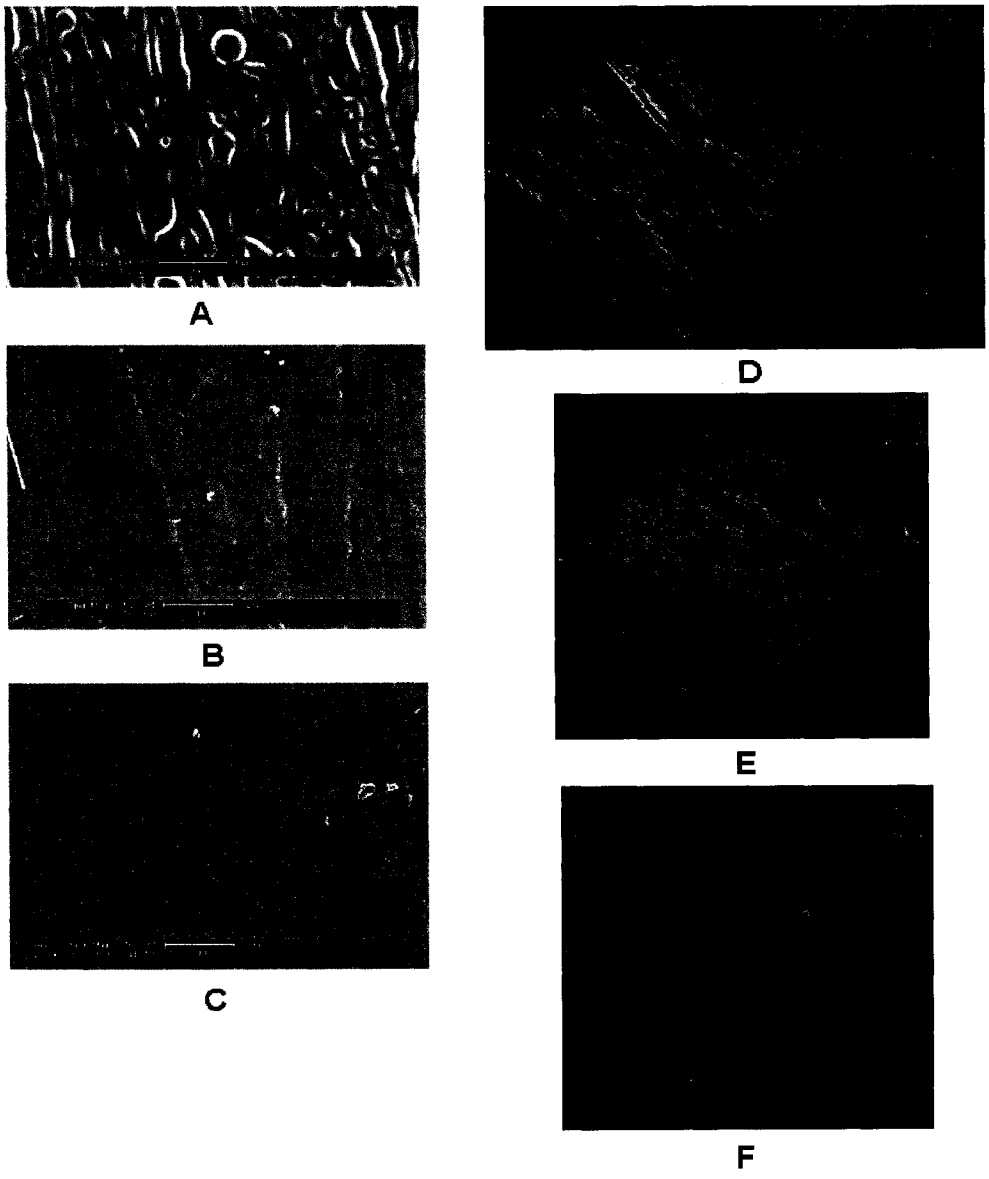

Figure 3.3 SEM and AFM scans of landed trypsin.

Panel A: SEM of soft-landed trypsin. Panel B: SEM of reactively landed trypsin after washing by four solution assays. Panel C: SEM of blank plasma treated surface. Panel D: AFM of soft-landed trypsin. Panel E: AFM of reactively landed trypsin after washing by four solution assays. Panel F: AFM of blank plasma-treated surface.

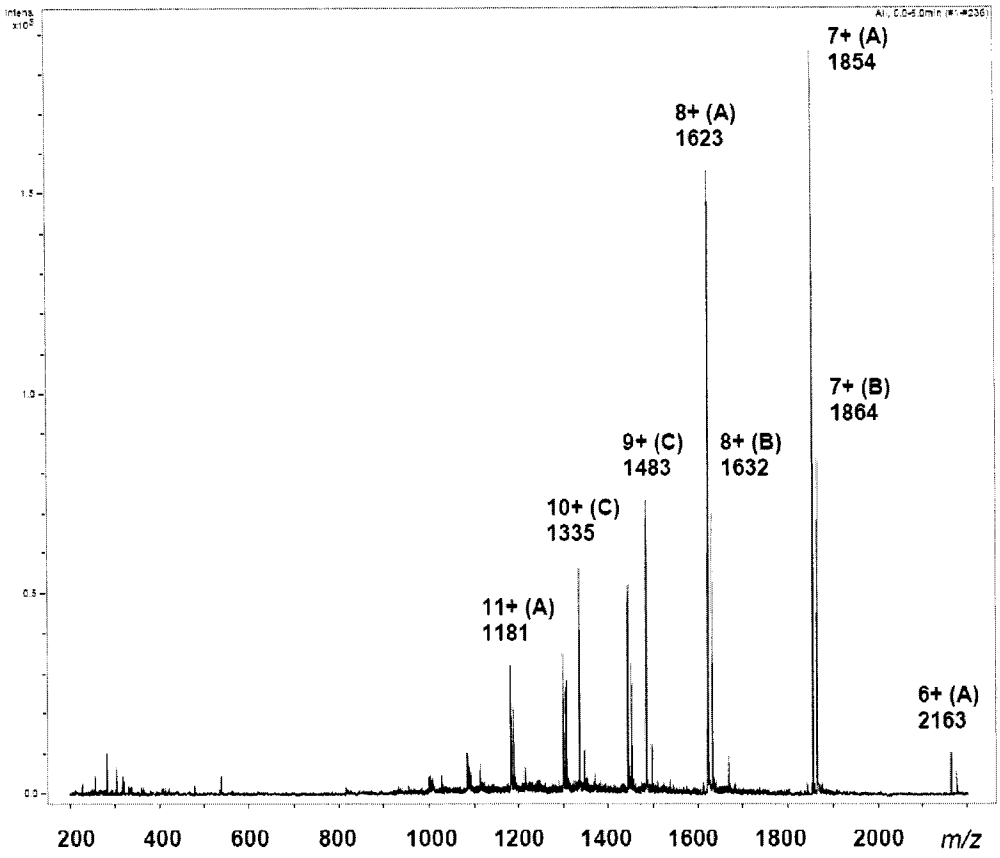

Figure 3.4 Electrospray mass spectrum of streptavidin.

Spectrum was obtained on a Bruker Esquire quadrupole ion trap mass spectrometer with electrospray ionization. It shows charge distribution characteristic for ESI-MS of proteins.

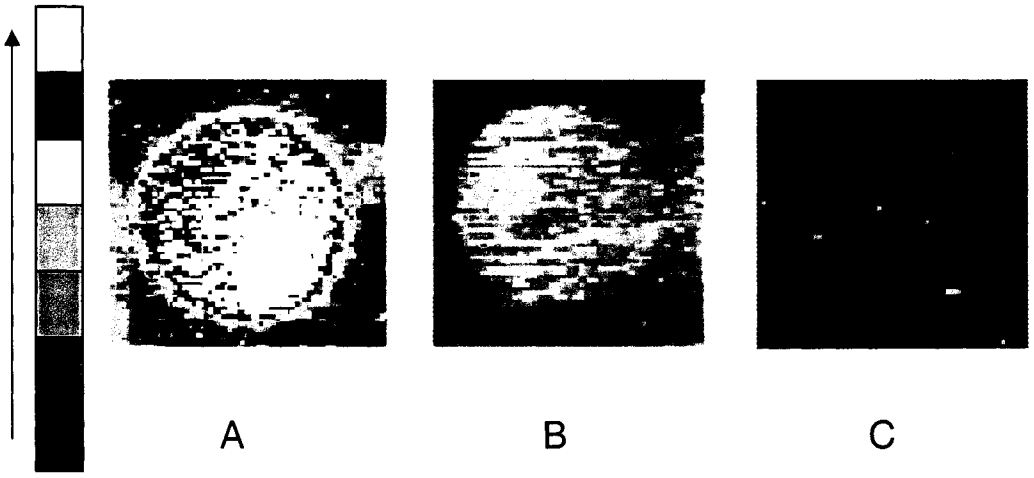

Figure 3.5 Surface fluoresecence of landed AlexaFluor-488 streptavidin.

Fluorescence scans of (A) soft landed AlexaFluor-488 streptavidin conjugate directly after the soft landing; (B) the same surface after 375 min of washing in PBS buffer; (C) blank surface that was treated with oxygen plasma, exposed to a solution of OregonGreen-488 streptavidin conjugate in PBS and rinsed with water.

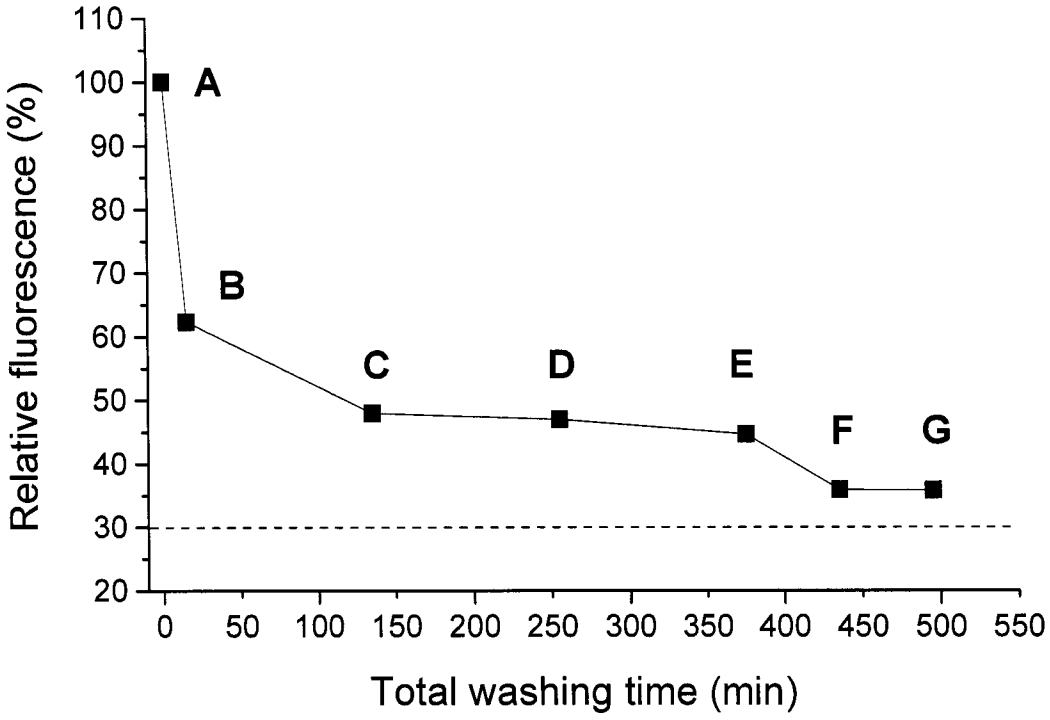

Figure 3.6 Decrease of relative fluorescence due to consecutive washing steps.

A: Intact soft landed sample = 100%; **B**: After 15 min washing with 100 µL of PBS; **C,D,E**: After additional washings with 100 mL of PBS for the indicated time periods; **F,G**: After additional washings with 100 mL of 25 mM sodium hexyl sulfate in PBS for the indicated time periods. The dashed line at 30% indicates the background level.

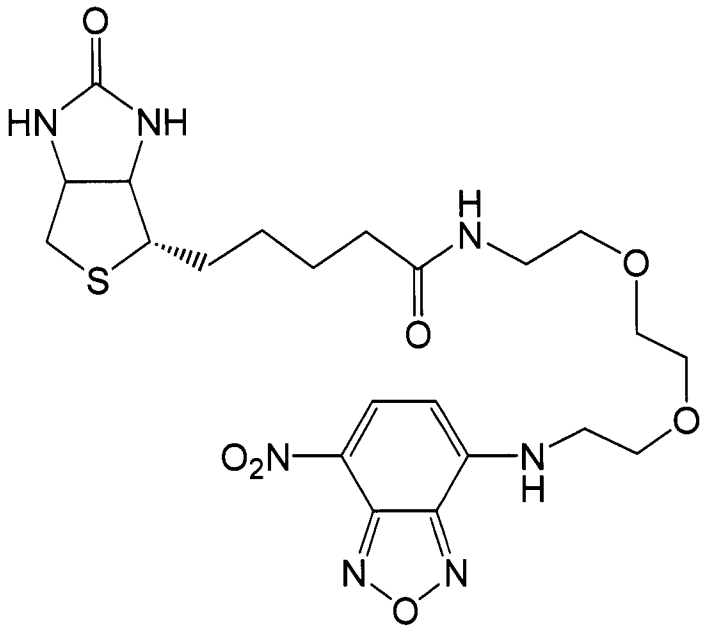

Figure 3.7 Chemical structure of the Biotin-NBD conjugate.

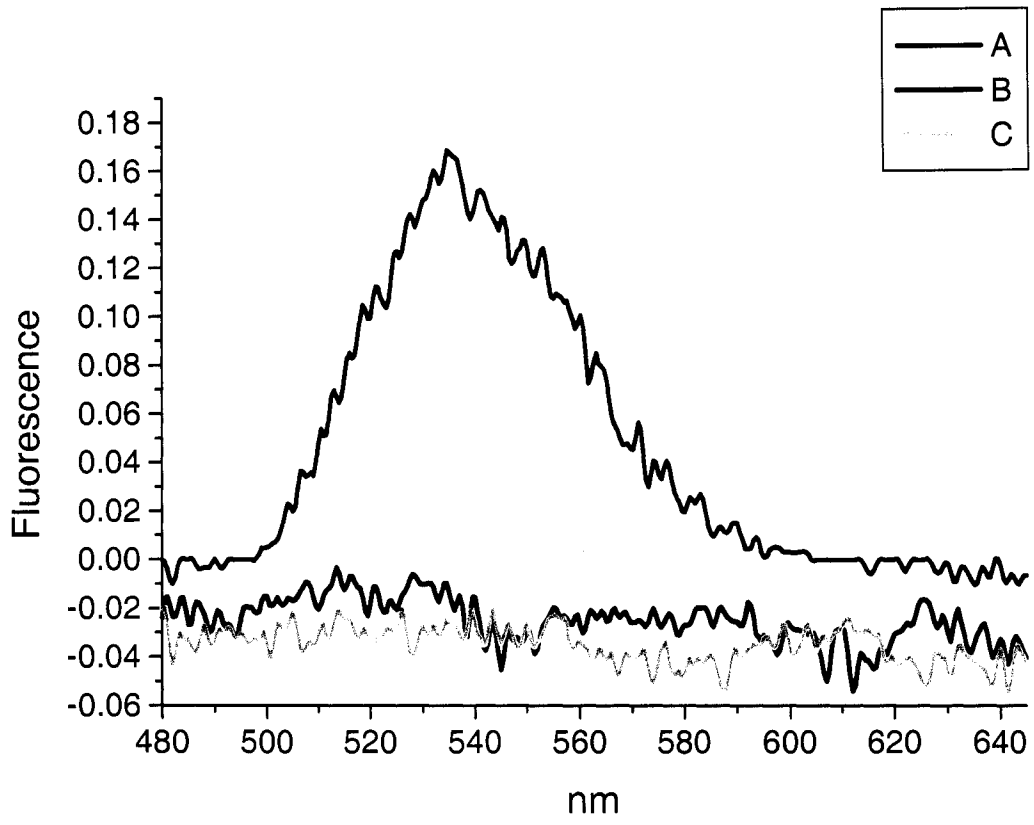

Figure 3.8 Fluorescence spectra of displaced Biotin-NBD conjugate.

Fluorescence spectra (excitation at 462 nm) of (A) Biotin-NBD displaced by pure biotin from a complex with soft-landed, surface-immobilized streptavidin. (B): Wash solution from a blank surface that was previously exposed to Bio-NBD. (C) Saturated aqueous biotin solution. Equal volumes of methanol (0.5 mL) were added to each solution prior to measurements to enhance fluorescence.

Table 3.1 XPS Analysis of Metal Surfaces

Composition (in atomic %)

Element	Blank	Landed Trypsin
C	49.6[a]	74.5
O	43.4	17.5
N	-[b]	5.6[c]
Fe	5.0	0.5
Cr	0.7	-[b]

[a] The high amount of carbon detected by XPS on blank surface is due to the hydrocarbon contamination that originates from the mechanical pumps oil and other sources in the surrounding lab environment. This carbon contamination can not be avoided because the instrument operates at medium vacuum only. In addition, the XPS measurements were done in a separate facility and every prepared sample spent significant time at ambient pressure conditions.
[b]Not detectable.
[c]A monolayer of pure protein would give 12-14%

3.6 Notes to Chapter 3

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CHAPTER 4: SOFT LANDING PREPARATIVE YIELDS

4.1 Introduction

The separation requirements of molecular and cell biology research in the nineties were the basic motivation responsible for restoring the interest in the idea of preparative mass spectrometry. Nevertheless, the community of mass spectrometric scientists still objects mainly three things. The first is the small yield of soft landing. In the letter[1] to the *Journal of Mass Spectrometry* in June 1999 Siuzdak and coworkers stated that the efficiency of electrospray preparative mass spectrometric experiments is approximately $1{:}10^7$ (for every 10^7 molecules in solution that are sent into the electrospray mass spectrometer, only one molecule can be recovered after soft landing). Such a low yield, authors of the letter argue, represents a significant limitation for ESI-MS as a preparative tool, and it will have to be compensated for by reintroduction of the wasted sample (from the ion source and analyzer) into the instrument. The second objection is that only a fraction of the landed ions stays intact. Fragmentation (or polymerization on the other hand) of landed material represents a serious problem because not only does it reduce the yield but mainly because it provides a new contamination of the original sample. And finally, the third problem is that MS can not directly separate isomers. Although there is an extensive literature on application of MS to organic stereochemistry[2] the experiences from analytical MS will not be easily transferable for the purposes of preparative MS. That is why it is necessary to accept this very last objection as given by the nature of separation process in MS. However, advances can be achieved to overcome the first and second objection.

To make soft landing of gas-phase ions a practical procedure, it is first necessary to determine and possibly optimize the yield of the material that is deposited on and recovered from the solid surface.

4.2 Preparative soft landing of low molecular mass compounds

Five compounds of medium molecular mass were used to investigate soft landing yields: the amino acid lysine (**1**), the dipeptide Phe-Leu (**2**), the tripeptide conjugate Val-Leu-Lys-*p*-nitroaniline (**3**), hexamethylpararosaniline chloride (crystal violet dye, (**4**) and biotin conjugate (**5**). The first three analytes form singly protonated ions by electrospray at *m/z* 147, 279, and 479, respectively. Dye **4** is an intrinsically ionic compound that in electrospray provides a cation at *m/z* 372. The biotinylated conjugate **5** forms $(M + H)^+$ and $(M + Na)^+$ ions at *m/z* 538 and *m/z* 560, respectively. The chemical structures are shown in Figure 4.1.

4.2.1 Lysine

Electrospray ionization of solutions of **1** produces mainly singly protonated ions at *m/z* 147. For soft landing, a 3.4×10^{-4} M solution of **1** in 9:1 methanol-water was electrosprayed at 6.7 μL/min flow rate and the ions were deposited without mass separation at 5 and 50 eV landing energy for 195 min each. In each case this corresponded to 4.4×10^{-7}mol of lysine that was electrosprayed. The soft-landed material was recovered from the surface into 100 μL of deionized water, diluted with 200 μL of methanol, 10 μL of 4×10^{-3} M solution of lysine ^{15}N was added as an internal standard, and the samples were analyzed by ESI-MS. Because the internal standard is chemically identical to **1**, it can be presumed that both have practically identical responses upon ESI-MS. From the ESI-MS analysis, it can be deduced that the total amounts of **1** recovered form soft-landing were 3.9×10^{-9} and 3.3×10^{-9} mol for the 5 eV and 50 eV landing energies, respectively. Hence the total, solution-to-solution, recovery yield of soft-landing **1** was 0.9% and 0.7% respectively.

Two points are noteworthy here. First, the ESI mass spectra of the soft-landed material were similar to the reference spectrum of **1**. In particular, the spectra of the soft-landed and recovered lysine did not show fragments that would indicate decomposition by decarboxylation, deamination, side-chain cleavage, nor higher

molecular mass condensation products. Second, the total yields achieved in these soft landing experiments exceed the previous reported results more than *ten thousand fold* (Siuzdak et al.[1]). These features and the soft landing mechanism will be discussed later in this chapter.

4.2.2 Phe-Leu

Peptide **2** was electrosprayed from a 3.6×10^{-4} M solution and the ions were soft-landed on a freshly plasma treated stainless steel surface at 5 and 50 eV. The soft-landed material was recovered from the surface as described for lysine and analyzed by ESI-MS using 8×10^{-9} mol of Phe-Val as an internal standard. Although Phe-Val is not chemically identical to **2**, it can be presumed that ionization efficiencies of these two homologues are very similar to give similar responses in ESI-MS[3]. The total, solution-to-solution yields were 0.9% and 0.8% for **2** soft-landed at 5 and 50 eV, respectively. Again, the ESI mass spectra of the soft landed material did not reveal decomposition or condensation products of **2**.

4.2.3 Val-Leu-Lys-*p*-nitroaniline

Peptide conjugate **3** was electrosprayed from two solutions at 1×10^{-4}M and 1×10^{-5}M in 9:1 methanol-water. The deposition time for soft landing was adjusted for the different concentrations such that it was 120 min for the more concentrated sample (6×10^{-8} mol electrosprayed) and 240 min for the less concentrated one (1.2×10^{-8} mol electrosprayed). The landing energy for singly charged ions was 20 eV in each case. The soft landed material was recovered in solution, as described for **1** and **2**, and analyzed by ESI-MS. Tripeptide Val-Val-Lys was used as an internal standard and the response to it and **3** was determined from a calibration curve. The recovered amounts of soft landed **3** were 4.3×10^{-10} and 2.3×10^{-10} mol when sprayed from the 10^{-4} M and 10^{-5} M solutions. This corresponds to 0.7% and 2% yield, respectively.

4.2.4 Crystal Violet

A 4×10^{-4} M solution in 9:1 methanol-water, corresponding to 6×10^{-7} mol of **4**, was electrosprayed and the ions were soft landed on a freshly plasma treated stainless steel surface at 5 and 50 eV. The soft-landed material, which formed a visible purple spot on the stainless steel plate, was washed with 100 μL of methanol, diluted with 600 μL of deionized water, and analyzed by UV/visible spectrophotometry at 586 nm. The recovered amounts were 1.3×10^{-9} and 0.75×10^{-9} mol for the 5 and 50 eV landing energy, respectively, corresponding to 0.1 and 0.2% total yields.

4.3 Discussion of Soft Landing Yields

The yields of isolated material upon ion soft landing (Y) can be described as being composed of fractions representing the efficiencies of ion formation in electrospray (α_{ESI}), ion transport into and through the vacuum system (β_{trans}), non-destructive ion discharge on the surface ($\gamma_{discharge}$), and elution from the surface ($\delta_{elution}$):

$$Y = 100 \times \alpha_{ESI} \times \beta_{trans} \times \gamma_{discharge} \times \delta_{elution} \qquad (4.1)$$

It can be presumed that β_{trans} remains constant for **1-3** and **4** given the identical charges of the transmitted ions and the absence of mass discrimination effects in the ion optics system. The tremendous increase in Y over previous reports[4] is most likely due to an improved ion transmission through the novel instrument which achieves ion currents on the order of several 10^{-9} A in the vacuum region where soft landing takes place[5,6]. Since the electric currents produced by electrospray of electrolyte solutions in the concentration range used here (10^{-4}M) are on the order of 10^{-7} A[7,8], the 10^{-9} A currents of gas-phase ions measured in vacuum indicate β_{trans} on the order of a few percent, but independent of the ion nature.

The $\delta_{elution}$ are presumed to be closely similar for **1-3** and **4**, because they are all polar compounds with excellent solubility in aqueous methanol. Thus, the differences in the isolated yields can be attributed to different α_{ESI} and $\gamma_{discharge}$. The relative ionization efficiencies in electrospray were determined for **2** and **4** on the ion trap mass spectrometer as follows. In separate runs, 10^{-6}M solutions of **2** and **4**, a mixture consisting of 10^{-6} M **2** and 10^{-6} M **4**, and a mixture consisting of 10^{-7} M **2** and 10^{-7} M **4** were electrosprayed and the ion intensities at *m/z* 279 for $(\mathbf{2} + H)^+$ and *m/z* 372 for $\mathbf{4}^+$ were recorded. In each case, the response to **4** was greater than for **2**, giving a mean ion intensity ratio of $[\mathbf{4}^+]/[\mathbf{2} + H]^+ = 8.1 \pm 0.6$. Note that both **2** and **4** are ionized non-destructively by ESI, and so the analyte current is carried by the corresponding ions at *m/z* 279 and 373 and their pertinent stable isotope satellites which are transmitted and detected with similar efficiencies. Hence, the 8.1 ratio reflects a greater ionization efficiency of **4**. The relative soft landing yields and ionization efficiencies can be used to extract the relative efficiencies for non-destructive ion discharge on the plasma-treated metal surface as shown for ions deposited at 5 eV.

$$\frac{Y(\mathbf{4})}{Y(\mathbf{2})} = \frac{0.1}{0.9} = \frac{8.1\gamma_{\mathbf{discharge}}(\mathbf{4})}{\gamma_{\mathbf{discharge}}(\mathbf{2})} \qquad (4.2)$$

$$\gamma_{\mathbf{discharge}}(\mathbf{2}) = 73 \times \gamma_{\mathbf{discharge}}(\mathbf{4}) \qquad (4.3)$$

These show that proton-carrying cations of $(\mathbf{2}+H)^+$ are discharged substantially more efficiently than the aromatic cation of **4**. The same ratio becomes lower $\gamma_{discharge}$ (**2**) = 32 × $\gamma_{discharge}$ (**4**), for landing at 50 eV.

It should be noted that the amino acid and peptide analytes may form proton-bound dimers or higher oligomers when electrosprayed at 10^{-4}M concentrations used in soft-landing experiments. The extent of this oligomer formation is difficult to

assess, because our soft-landing instrument does not have a mass analyzer, and the concentrations used for soft landing are too high for an analytical mass spectrometer. Proton-bound dimers or oligomers would increase the efficiency of analyte transfer when based on the ion current measured at the landing plate. However, it was found that decreasing the concentration of **3** ten fold resulted in a more than two-fold *increase* of soft-landing yield. Since dimer formation should scale with the square of analyte concentration, the increased yield at lower concentrations indicates that dimers and oligomers may not be important in analyte transfer into the vacuum system for soft landing.

In spite of the much greater discharge efficiencies of peptide ions compared to that of **4**, the 0.7-2% isolated yields do not allow one to assess the fraction of peptide ions that underwent chemical decomposition upon soft landing but remained chemically tethered to the surface and so would be undetectable in the rinse solution. At both 5 and 50 eV, the gas-phase ions impinge on the surface with kinetic energies that greatly exceed their internal (rovibrational) enthalpy. For example, gas-phase (**2** + H)$^+$ with 123 internal degrees of freedom is estimated to have an internal enthalpy equal to 0.18 × 123 × RT = 0.57 eV at 300 K [9]. This indicates that a full conversion of the ion kinetic energy into its internal energy could drive chemical reactions and decomposition on the plasma treated metal surface. To gain some insight into this issue, it was interesting to investigate soft landing of gas-phase ions where damage in the material on the surface can be detected by changes in biological or spectroscopic properties.

4.4 Soft and Reactive Landing of Fluorescence-Labeled Biotin Conjugate

A synthetic biotin conjugate with fluorescence moiety was used. This compound consisted of two functionally orthogonal reporter groups that were connected by a hydrophilic polyethylene glycol diamine linker (**5**). One reporter group was a biotin moiety that can be recognized by the non-covalent and highly

specific bioaffinity interaction with streptavidin. The other reporter group was a nitrobenzofurazan (NBD) fluorescent label. Thus, chemical damage in the biotin moiety upon ion soft landing should impair or prevent recognition by streptavidin, whereas damage in the fluorescent label should affect the fluorescence yield, emission wavelength, or both.

Conjugate **5** forms singly protonated (*m/z* 538) and singly sodiated (*m/z* 560) ions in a 1:6 ratio when electrosprayed from a methanol solution. The protonated and sodiated ions from **5** were soft landed on a freshly plasma treated stainless steel surfaces at 0, 5, 10, 20, 30, 40, and 50 eV. At all landing energies the soft-landed material formed a visible yellow spot (Figure 4.2). The surfaces were examined by a fluorescence scanner and all spots were found to exhibit fluorescence well above the background level of the scattered excitation laser beam (Figure 4.3a). The soft-landed material was then washed with 100 μL of methanol, and the solution was stored at -20 °C for further experiments (see below). The washed surfaces were scanned but no fluorescence above the background level was detected (Figure 4.3b). In the next step, the same washed surfaces were exposed to a solution of OregonGreen-488 labeled streptavidin (1 mg in 3 mL of PBS), rinsed by water and scanned (Figure 4.3c).

The samples of **5** that were prepared at landing energies <40 eV did not show any fluorescence at 570 nm that could be assigned to affinity-bound OregonGreen-488 labeled streptavidin. However, fluorescence from the landing spot was detected from samples where ions **5** were deposited at 40 and 50 eV (Figure 4.3c). That this fluorescence originated from OregonGreen-488 labeled streptavidin was confirmed by using a 570 ± 15 nm filter with a wavelength bandwidth matching the emission range for OregonGreen-488.

These experiments indicated that ions of conjugate **5** that were soft-landed at kinetic energies up to 30 eV did not form chemical bonds to the surface and the landed material was washed off with solvent. In contrast, a fraction of ions that were

landed at ≥ 40eV was immobilized on the surface but retained an intact biotin moiety that was recognized by streptavidin.

There are two possibilities that can explain the absence of fluorescence from surface-bound **5.** The first is that the fluorescence of the NBD chromophore on the surface is quenched by strong interactions with electrons in the metal conductivity band[10]. Although it can be expected that the plasma-treated metal surface is covered by several hundreds nm of oxide, the separation from the metal of the NBD fluorophore in soft-landed **5** may not be sufficient to prevent quenching. Because fluorescence quenching decreases with the third power of the separation between the fluorophore and the metal surface[10a], it must be much weaker in the OregonGreen-488 chromophores bound to the large streptavidin moiety where at least some chromophores are substantially remote from the metal surface. A second explanation is simply that NBD chromophores in a fraction of the soft-landed **5** were chemically damaged and lost fluorescence capability, so that the total fluorescence of the soft-landed sample was below the detection limit of the scanner.

To characterize and quantify the wash solutions of soft landed **5,** they were diluted with 600 μL of methanol, and analyzed by UV/visible spectrophotometry at 462 nm and by fluorescence spectrophotometry at 539 nm (excitation at 462 nm). Calibration curves were determined from standard solutions of pure **5** in methanol. The results of these measurements, together with total solution to solution landing yields, are summarized in Table 4.1. The amounts of recovered **5** and the recovery yields show some fluctuations over the 0-50 eV range of landing energies. The fluctuations are in part due to the usual ion current fluctuations inherent to electrospray ionization. In addition, the gradual decrease over the period of 1-2 h of the ion current that was monitored on the soft-landing metal plate. Interrupting the electrospray for a few minutes and restarting it again resulted in a renewal of the ion current close to the original value measured at the landing plate.

These observations indicate that the charge brought to the plate surface by the impinging gas-phase ions was not immediately discharged into the metal and

resulted in partial charging of the surface that scattered the impinging ions away from the plate and thus lowered the landing yield. The imperfect discharging may be due to the relatively high proportion of $(M + Na)^+$

Note that both absorption and fluorescence measurements gave virtually identical amounts of the recovered material. Since free NBD in solution does not show fluorescence, the identical yields from absorbance and fluorescence measurements are evidence that no NBD groups were cleaved from **5** upon soft landing to be washed into solution with the intact conjugate. In accordance with this finding, the ESI-MS spectrum of **5** landed at 50 eV and washed in methanol did not show ions that could be assigned to decomposition products of **5**. An MS/MS spectrum of the $(M + Na)^+$ion (*m/z* 560) from the soft-landed **5** was identical to the reference spectrum of the same ion obtained by ESI of the stock solution of **5** (Figure 4.4).

Samples of soft-landed **5** were also examined for bioaffinity to streptavidin of the conjugated biotin moiety. Solutions of soft landed and washed **5** (300 μL aliquots of the 700 μL stock solutions, see above) were mixed with 200 μL of water and 100 μL of streptavidin-agarose beads that were previously washed with methanol and dried. For reference, the same procedure was applied to all calibration solutions that were used in previous absorption and fluorescence measurements of total yields of soft landed conjugates. After 20 h exposure at room temperature under occasional stirring, the beads were separated by centrifugation and the supernatant was examined for free conjugate by fluorescence. The fluorescence of all solutions (soft-landed samples as well as calibration solutions) was lower than 0.1% of the original value that was measured before capture on streptavidin-agarose beads. This indicated that most of the conjugate was captured by the streptavidin beads. Because the non-covalent interaction with streptavidin is highly specific for biotin, the quantitative capture of **5** is a key proof that the soft-landed **5** retained an intact biotin group.

That a specific biotin-streptavidin interaction was indeed involved was confirmed by recovery experiments. The streptavidin beads with captured **5** were

separated by centrifugation and rinsed three times (methanol, water, and methanol) to remove nonspecifically adsorbed conjugate. The beads were dried, and **5** was eluted with 250 μL of saturated solution of free biotin for 3 days at room temperature with frequent mixing. The concentration of **5** in solution was determined by fluorescence at 539 nm according to a calibration curve that was constructed from the original calibration solutions that received the same treatment, that is, they were captured by streptavidin, washed, and eluted with a biotin solution. Table 4.1 shows that the results are in general consistent with those from the initial determination of soft landing yields for **5**.

In summarizing the foregoing results, it was showed that a biotin-linker-NBD conjugate can be soft landed and reconstituted into solution with both orthogonal functions (fluorescence and biotin group) intact. The calculated solution-to-solution soft landing yield is comparable to the yields obtained for peptides and other medium-size molecules. At landing energies greater than 40 eV, a fraction of conjugates is immobilized on the surface that exhibits biotin bioaffinity, but not fluorescence.

4.5 Mechanisms for Soft Landing on Plasma-Treated Metal Surface

The aforementioned findings, that intact peptides, ions, and biotin conjugates are produced by landing substantially hyperthermal gas-phase ions on plasma treated metal surfaces, raise the questions of energy deposition, charge neutralization, and chemical reactions upon ion-surface interaction. The last two processes are most likely linked and affected by the chemical composition of the surface. The plasma-oxidized surface of the landing plates was analyzed by XPS and showed mainly Fe_2O_3 and Cr_2O_3 in a molar ratio that was close to the bulk metal content of Fe and Cr in 316L stainless steel. The distribution of the metal oxides was measured at three different points on the plasma-treated surface that showed excellent homogeneity. Metal oxides are amphoteric and can both mediate proton transfer from the arriving

cations, and bind to the polar groups in the peptide and conjugate ions landed on the surface.

From the total charge of ions arriving on the surface, it is clear that the ions must be efficiently discharged to allow soft landing of several layers of material. From simple Coulomb law calculations it follows that even a monolayer of surface charges at a density of one elementary charge per 50 $Å^2$ would produce an electric field on the order of 10^8V/m that would be impenetrable to gas-phase ions of 5-50 eV kinetic energy. For example, soft landing of 5.8×10^{-9}mol of ions, as measured for $(Phe\text{-}Leu + H)^+$ (*vide supra*), brings in 5.6×10^{-4} C of charge which cannot be accommodated on the small landing spot of an area of 23-28 mm^2.

It is reasonable to conclude that ion discharge on the surface is mediated by proton transfer from the arriving cations to the metal oxide layer. This is coupled with protonated oxide reduction, e.g. $Fe^{3+} \rightarrow Fe^{2+}$, by electron transfer from the conductance band of the bulk metal. This assumption is based on the following arguments.

First, it is well known from mass spectrometry that discharge of peptide cations by electron capture results in substantial (20-80%) fragmentation of the disulfide and N-Cα bonds.[14] None of that is observed in soft landing of peptide ions on oxidized metal surfaces where only intact molecules are detected after washing from the surface. Therefore, ion discharge on the surface by direct electron transfer from the bulk metal is unlikely to be an important process.

Second, substantially lower yields were observed for soft landing and isolation of **4**, and to some extent also for $(\mathbf{5} + Na)^+$, which cannot discharge by proton transfer, because they lack prototropic groups. Furthermore, soft landing of **4** ions on clean (non-oxidized) metal surfaces results in a further 100 fold decrease of the yield of isolated **4** and the eluate shows mainly decomposition products[11]. All these results point to an acid-base prototropic mechanism for discharging peptide ions on the oxidized metal surface.

Third, surface charging effects were observed for soft landing of ions that contained a high proportion of $(M + Na)^+$ species. This is consistent with the lower mobility of Na^+ ions and hence slower transport of the charge from the upper layers of soft-landed ions to the metal oxide layer.

Finally, it is worth mentioning that although surface immobilization of soft-landed ions was observed only at relatively high kinetic energies, e.g. > 40 eV for **5**, the isolated yields of material were practically independent of the ion landing energy within this range for all compounds we investigated. These results indicate that kinetic-energy driven reactions with the surface metal oxide layer occur only in the first few monolayers of soft-landed organic ions. This double layer of metal oxide and the material deposited by soft landing can be thought of as a cushion that is able to dissipate the impact energy of the impinging hyperthermal ions and also to mediate proton transfer for efficient discharging. The compounds used in the present work to establish soft-landing yields do not allow facile quantification and characterization of the surface-immobilized material. In the accompanying paper we use large biomolecules to probe and quantify the chemical and spectroscopic properties of immobilized soft-landed ions.

4.6 Modification of electrospray ion source

In order to investigate if an instrumental modification has an impact on absolute soft landing yields the, conventional electrospray ion source was rebuilt and replaced with microelectrospray.

It consists of a microspray ion source (PicoTip emitter, 30 um d. with P200P coating, New Objective, Woburn, MA) that is positioned ca. 2 mm from the counter-electrode capping the transfer capillary. The PicoTip emitter is mounted in Velco Union and connected with high voltage power supply (Figure 4.5).

Samples were collected for 30-150 min, then the collector plates with landed material were removed from the vacuum system, the soft landed material was washed with 100-300 uL of solvent, and analyzed as shown in Table 4.2. A new

analyte, α-Dansyl-L-arginine hydrochloride (**6**) (Figure 4.6), was added to the previously used group of compounds. Dansyl-arginine is easy to quantify by the fluorescence of the dansyl group.

Samples for ESI-MS analysis were washed with a 90/10 methanol-water mixture. Samples for fluorescence spectroscopy measurements were washed with pure methanol and diluted as needed. Lysine (**1**) was quantified by electrospray ionization-mass spectrometry by comparing the intensity of the $(M + H)^+$ peak with that of the ^{15}N-lysine internal standard. Phe-Leu (**2**) was quantified by ESI-MS by comparing the intensity of the $(M + H)^+$ peak with that of Phe-Val internal standard. Again, Phe-Leu and Phe-Val have similar polarities, basicities, and molecular mass and are expected to have very similar ionization and detection efficiencies[3] even if the elctrospray is replaced with microspray. Conjugates dansyl arginine (**6**) and biotin-NBD (**5**) were quantified by fluorescence spectroscopy in solution using standard calibration curves.

The fluorescence-labeled conjugate **5** forms a visible spot on the landing plate, as shown in Figure 4.2. The time dependence of the amount of recovered **5** and the soft-landing yield was studied after 30, 90, and 120 min. deposition. Visual inspection of the landing spot (5-6 mm diameter, Figure 4.5) indicates an increasing amount of **5** deposited on the target at longer landing times. Quantitation by fluorescence assays of recovered **5** shows that the total amount of soft-landed and recovered **5** increases with the deposition time in a roughly linear fashion, as expected for an unimpeded constant mass flow to the target. (Figure 4.7). This also indicates that the overall soft-landing yield, expressed as %[mol recovered]/[mol electrosprayed], does not critically depend on the deposition time within the low nmol range of our measurements. The results are summarized in Table 4.2.

Is further improvement of soft-landing yields to 10-20% and beyond possible? The current improvement is mainly due to the increased efficiency of electrospray ionization that achieves higher currents of gas-phase ions at lower

solution flow rates in microspray. [11] This probably results from a more efficient collection of microdroplets to enter the capillary for ion transfer to the vacuum system, due to the fact that the PicoTip emitter can be placed within 2 mm of the capillary atmospheric side end (Figure 4.5). The ion currents measured in the vacuum system (1-2 nA) are well within the transmission limit of the ion funnel lens, which is close to 13-16 nA for the lens of this design.[6b,5] Ion transmission through an octopole guide coupled to a funnel lens is very efficient (90-95%)[6a] and the ions impinging on the metal plate are believed to be trapped with nearly 100% efficiency due to the electrostatic potential applied to the collector electrode. The yield of ion discharge on the metal oxide surface is an unknown quantity.

Thus, it appears that further efforts at improving recovery yields in soft landing should focus on increasing the ionization efficiency in electrospray and the efficiency of droplet transport into the vacuum system.

4.7 Conclusions

Soft-landing of gas-phase ions from electrospray ionization of amino acids, peptides, and a biotin conjugate achieves preparatively significant yields (up to 6% of absolute solution to solution yield or, in absolute terms, quantities higher than nmol) when using dry plasma-treated metal surfaces. Soft-landed samples are isolated in nmol quantities by simply washing the surface with a pure solvent. Protonated ions are found to furnish higher yields of intact soft-landed compounds than do sodiated or quaternary aromatic ions. Ions soft-landed at 40-50 eV kinetic energies form a layer that is chemically tethered to the metal oxide surface. This immobilized layer retains some properties of the intact molecules, e.g., the ability to be recognized by a non-covalent bioaffinity interaction.

The absolute soft landing solution-to-solution preparative yields were significantly improved by improvement of the ionization efficiency, which was achieved by relatively simple instrumental modification. Thus, it appears that further efforts at improving recovery yields in soft landing should focus on increasing the

ionization efficiency in electrospray and the efficiency of droplet transport into the vacuum system. On the other hand, including a mass analyzer to select the ions to be soft landed is likely to decrease the ion transmission and overall yields.[12,13] Optimization of soft landing procedures to achieve yields exceeding 10-20% will depend on balancing both the instrumental and electrochemical aspects.

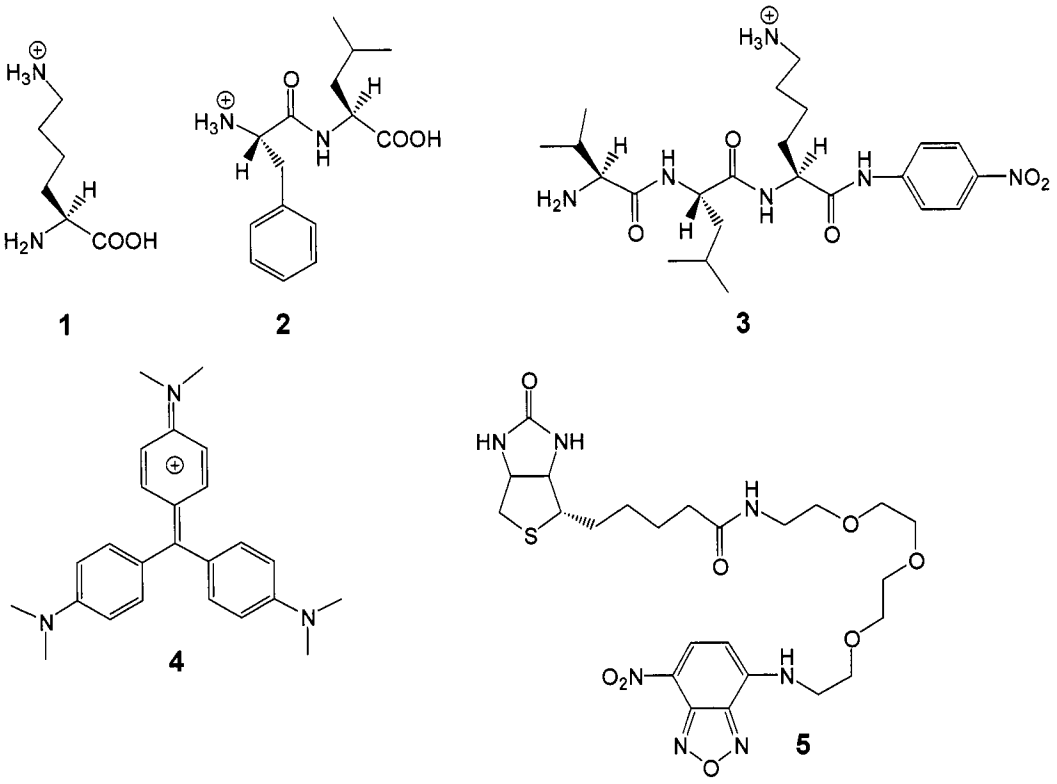

Figure 4.1 Chemical structures of compounds and ions used for soft landing.

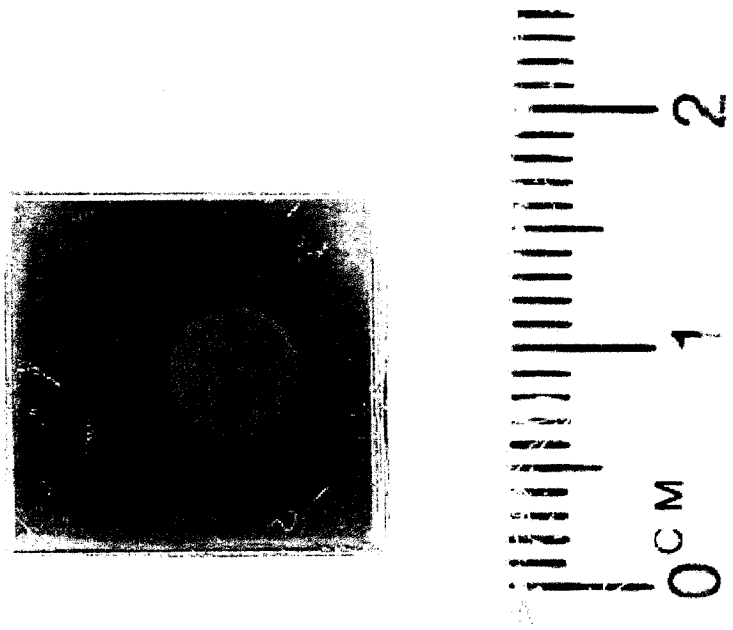

Figure 4.2 Photograph of the spot of landed material.

Photograph of the spot that was produced by soft landing of biotinyl-3,6,9-trioxaundecanediamine-NBD (Biotin-NBD) conjugate (**5**) soft-landed on plasma-treated stainless steel surface at 5 eV kinetic energy.

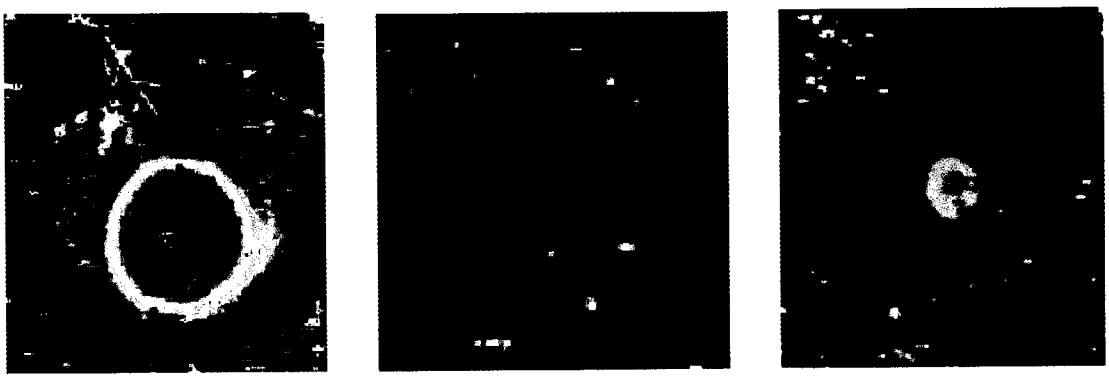

Figure 4.3 Surface fluorescence of landed biotinylated (Biotin-NBD) conjugate.

Fluorescence scans of biotinylated conjugate **5** that was soft-landed on a plasma-treated stainless steel surface at 40 eV kinetic energy. (A) Fluorescence before washing, (B) after washing with methanol, (C) after exposure to a solution of OregonGreen-488 labeled streptavidin.

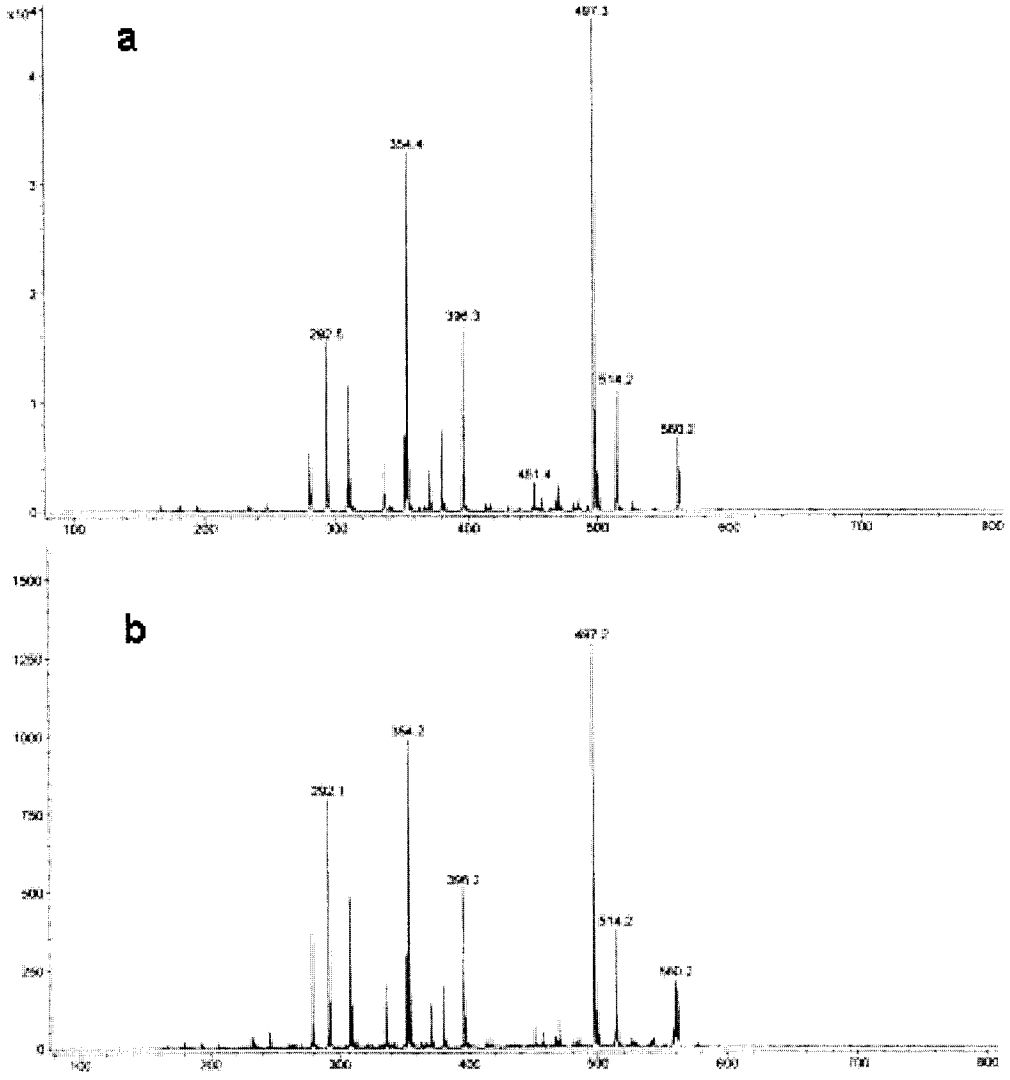

Figure 4.4 MS/MS CID fragmentation spectra soft landed and recovered biotinylated (Biotin-NBD) conjugate.

ESI-MS/MS spectra of of $(M + Na)^+$ ions at *m/z* 560 from (a) reference sample of **5** and (b) soft-landed and recovered sample of **5**.

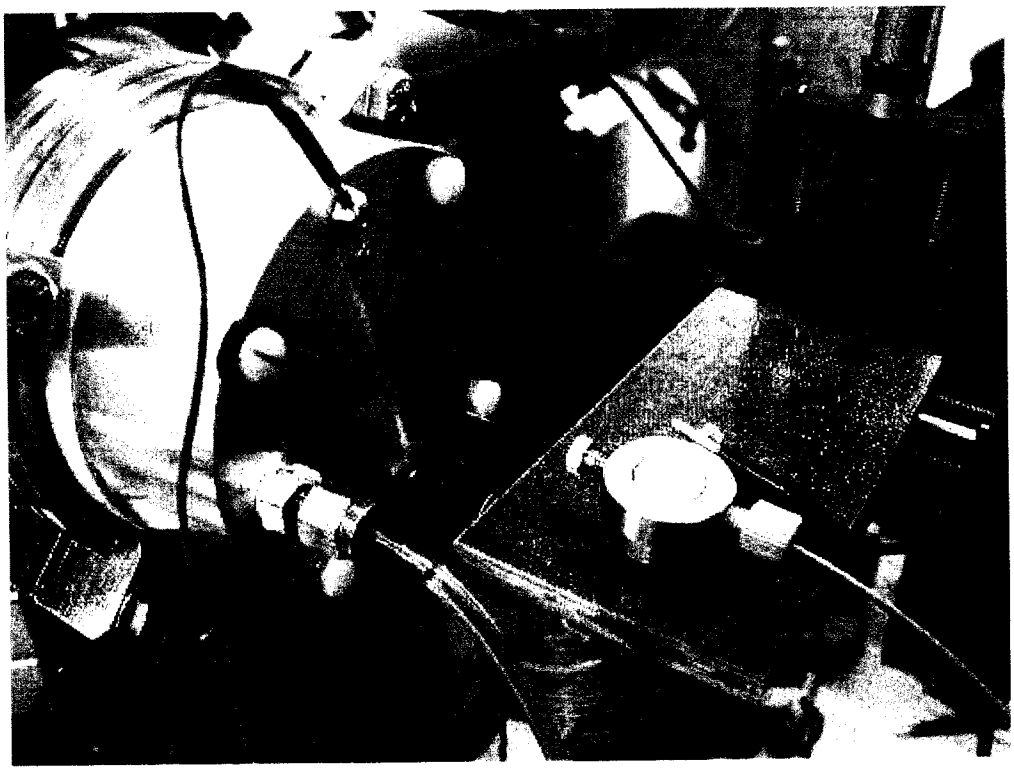

Figure 4.5 Photograph of the improved microspray ion source.

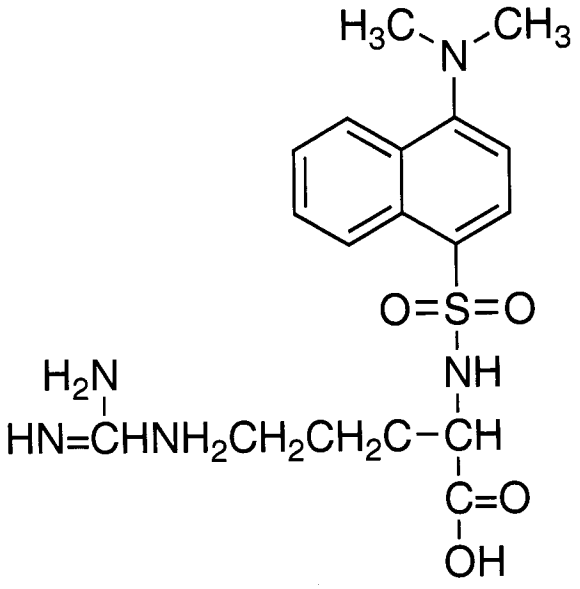

Figure 4.6 α-Dansyl-L-arginine hydrochloride.

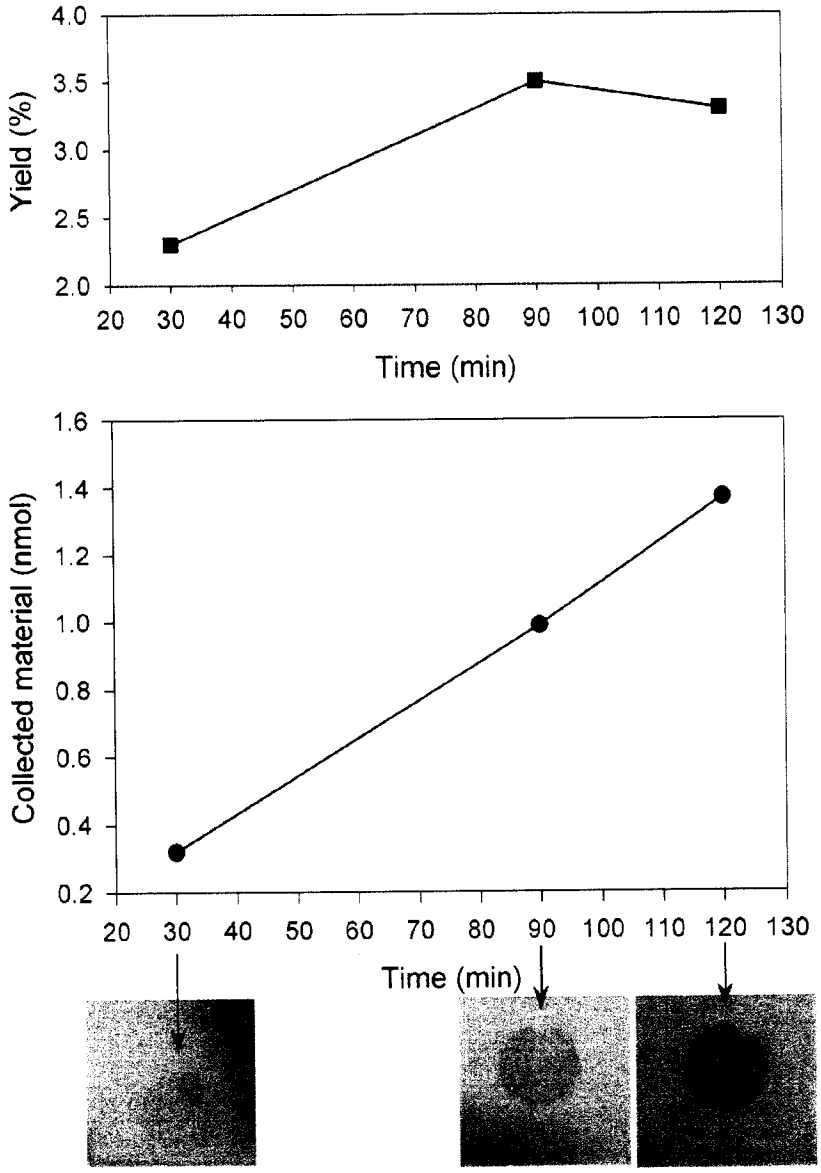

Figure 4.7 Soft landing yields with improved microspray ion source.

Yields (top), recoveries (middle), and photographs of landing spots (bottom) of fluorescent conjugate **5** at the indicated ion collection times. The landing spots have 5-6 mm diameters.

Table 4.1 Quantitative Analysis of Soft-Landed Biotin-NBD Conjugate (5).

Landing Energy (eV)	Recovered **5** (nmol) fluorescence	Recovered **5** (nmol) absorbance	Recovery yield (%)	Recovered **5** (nmol) after affinity capture/elution[c]
0	0.2[a]	0.2[b]	0.1	0.3
5	0.6	0.5	0.1	0.6
10	2.5	2.5	0.4	1.6
20	0.6	0.7	0.2	0.6
30	0.7	0.6	0.1	1.0
40	1.1	1.0	0.4	2.1
50	2.1	2.0	0.3	2.4

[a]From fluorescence measurements of soft-landed and washed **5**. [b]From absorbance measurements of soft-landed and washed **5**. [c]From fluorescence measurements of **5** that was soft-landed, washed into solution, and affinity purified by capture-elution on streptavidin-agarose beads.

Table 4.2 Improved soft Landing Yields obtained with microspray ion source.

Compound	Sprayed (nmol)	Recovered (nmol)	Yield (%)	Quantification method
Lysine (**1**)	2.7	0.17	6.3	ESI-MS[a]
Phe-Leu (**2**)	7.2	0.19	2.6	ESI-MS[b]
Biotin-NBD (**5**)	14	0.32	2.3	Fluorescence[c]
N_{α}-Dansylarginine (**6**)	3.4	0.20	5.9	Fluorescence[b]

[a]Using ^{15}N-lysine as an internal standard.
[b]Using Phe-Val as an internal standard.
[c]Fluorescence spectroscopy measurements in methanol solution at 535 nm.
[d]Fluorescence spectroscopy measurements in methanol solution at 515 nm.

Notes to Chapter 4

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CHAPTER 5: SURFACE ENHANCED RAMAN SPECTROSCOPY OF LANDED IONS

5.1 Introduction

In the previous two chapters, the phenomena associated with soft landing of gas-phase ions on oxide-coated metal surfaces have been studied using bioassays and fluorescence spectroscopy. While these methods provide valuable information on the biological activity of the surface-tethered material and the presence of intact fluorophores, they lack chemical specificity. In particular, the nature of bonding to the surface and chemical changes in the immobilized molecules are not revealed on a molecular level. Atomic force microscopy (AFM) has been used to visualize soft-landed protein on oxidized metal surfaces (Chapter 3), but in general AFM did not achieve sufficient spatial resolution to recognize fine structural details. X-ray photoelectron spectroscopy (XPS) and secondary ion mass spectrometry (SIMS) have also been used to provide the elemental composition[1,2] or mass of soft-landed species or their fragments.[3,4,5] However, a sensitive and structure-specific spectroscopic method for the investigation of soft-landed ions and molecules directly on the surface has been lacking so far. The discovery presented here represents a new approach to structure characterization of soft-landed species at low zeptomole levels using surface-enhanced Raman scattering (SERS).[6,7]

SERS provides information on Raman active vibrational modes in molecules that are in a close contact with electrochemically roughened or chemically etched metal surfaces,[8] vapor-deposited metal films,[9] or colloid particles of silver, gold or platinum.[10] SERS achieves an amplification of the Raman effect on the order of 10^6-10^8 fold. Under favorable circumstances SERS even allows detection of single molecules on gold nanocrystals.[10,11] The known shortcoming of SERS is that the preparation of SERS-active surfaces suffers from rather poor reproducibility, which is particularly true for colloid particles.[11a]

It was discovered that soft landing of cations on plasma-treated silver

substrates produces samples that reproducibly provide high quality Raman spectra. The spectra can be used to analyze the Raman active vibrational modes and thus to characterize the surface-bound molecules at low zeptomole detection limits. In this dissertation the first study aimed at structure elucidation of soft landed and immobilized polyatomic cations including organic dyes and nucleosides is reported

The plasma treatment of silver substrates was carried out under a constant flow of Ar/O_2 at a constant pressure of 0.25 Torr. The target was placed in the glass plasma chamber and exposed to air/Ar plasma at 0.25 Torr and 60 W for 15 minutes. The development of silver oxide was visible by the formation of a dull gray layer on the glossy metallic silver substrate. Most analytes were electrosprayed as methanol-water solutions at 30-300 μM concentrations in a positive mode under optimized conditions. The ion currents, as measured on the silver target, were typically 1-3 nA. The ions were deposited for 4 h to obtain 100-500 pmol of soft-landed material. Unless stated otherwise, the nominal landing energy for singly-charged cations was 40-50 eV as determined by applying a -40 to -50 V potential on the landing plate.

5.2 Soft Landing and Raman Spectra of Organic Dyes

Upon electrospray, crystal violet produces singly-charged $C_{25}H_{30}N_3$ cations at *m/z* 372 (CV^+). Previous experiments have shown that CV^+ survived soft-landing on plasma-treated stainless steel (Chapter 4). Soft landing of CV^+ on plasma-treated silver substrates, followed by spectroscopic analysis of the surface-bound material, produced the Raman spectrum shown in Figure 5.1. The spectrum obtained from landing CV^+ at near-thermal kinetic energy (Figure 5.1a) shows several intense Raman bands. Most of these bands are also present in the reference spectrum obtained for a 4 mM solution of CV^+ chloride in methanol (Figure 5.1b). Remarkably, very similar Raman spectra were obtained for CV^+ ions that were landed at higher nominal impact energies of 10, 50, and 250 eV (Figures 5.2-5.4). The Raman spectra showed only minor changes in intensity or band positions when the samples were exposed to multiple washings with methanol each lasting for up to

5 h and repeatedly exposed to the 514.5 nm excitation line. No photolytic bleaching of the surface-immobilized material was observed under the experimental conditions. Multiple blank and control samples were investigated to confirm the identity of the SERS active species. The Raman spectra of plasma-treated Ag surfaces showed no spectroscopic features. Furthermore, exposing the plasma-treated silver surface to 3 μM solution of CV^+ chloride for up to 15 h, followed by rinsing with solvent, produced no SERS signal from the surface (Figure 5.5). Other controls included dipping the plasma-treated Ag substrate into more concentrated CV^+ solutions for shorter periods of time or drying 2 nmol of CV^+ on the surface (Figure 5.6). None of these controls provided SERS signal of CV^+. In contrast, extended washing with methanol of soft-landed CV^+ samples caused no significant decrease of or changes in the SERS spectra (Figure 5.2). Thus, it can be concluded that the SERS spectra must correspond to CV^+ ions that were deposited from the gas phase and adhered to the plasma-treated silver surface.

The SERS of CV^+ was previously reported on island Ag films[12a] and on rough thin Ag films[12b]. These SERS spectra were obtained by dropping relatively concentrated CV^+ solutions (0.01mM-50mM) on different Ag substrates. They show same peaks as spectra obtained by soft landing that are presented here. However, studies on Ag films reported instability of acquired spectra in time, which was not observed in this work with samples prepared by soft landing.

The Raman active modes in both SERS and solution Raman spectra were assigned on the basis of a vibrational analysis using density functional theory calculations of CV^+ in the gas phase (Figure 5.1c) and aqueous solution.

The optimized structures of the ion are very similar in both media and show a propeller-like geometry of C_3 symmetry (Figure 5.7). Compared to the solution spectrum, the SERS of soft-landed CV^+ shows some differences in peak positions and relative intensities. The peak of the out-of-phase ring stretch (1622 cm^{-1}) is split into two peaks at 1630 and 1610 cm^{-1} in SERS. The fact that this mode is not degenerate in the surface-tethered ions may indicate that there are populations of

species that adopt different orientations with respect to the surface. The CV^+ band at 1372 cm^{-1} consists of the unresolved in-plane C—H bending mode and symmetrical N—C-ring-C—C stretching mode whose wavenumbers are calculated at 1352 and 1364 cm^{-1}, respectively. This band is notably broader in the SERS spectrum and is blue shifted to 1405 cm^{-1}. Blue shifts in the SERS spectra are also observed for the in-plane C—H bends (1181 cm^{-1} shifted to 1205 cm^{-1}) and symmetrical ring breathing modes (921 cm^{-1} shifted to 940 cm^{-1}). The nature of these shifts may in part be due to polarization of the surface species by the Ag^+ ions that are present in the metal particles. This hypothesis has been suggested by Otto *et al.*[13] where such a coordination was expected to shift the vibrational frequencies of adsorbates as in the case of ethylene.[13] The magnitude of such shifts for larger polyatomic compounds is currently unknown.

5.3 SERS Detection Limits

The lowest amount of immobilized CV^+ that can be detected on plasma-treated Ag substrate was determined by electrospraying and soft landing at 50 eV mixtures of CV^+ and Rhodamine B at ratios that were varied from 1:1 to 1:1000. CV^+ and Rhodamine B have very similar ionization efficiencies in electrospray[14a] and thus the relative flux of soft-landed species is close to their relative concentrations in solution. In all these measurements, the loosely deposited ions were thoroughly washed off with solvent, so that only the surface-tethered ions were analyzed. Both CV^+ and Rhodamine B dyes produced intense SERS spectra (Figure 5.8) that were distinguished by the band at 940 cm^{-1} that is unique for CV^+. The detection limit at 3 standard deviations of background was found at a 1:600 CV^+/Rhodamine B ratio. Taking into account the 0.8 μm^2 size of the spot that was irradiated by the excitation laser, and assuming close packing of the surface-bound ions of an 85 $Å^2$ cross section, we calculated the limit of detection for CV^+ at 2.5 zeptomoles. Note that the total amount of soft-landed material (~10^{-10} mol) was substantial greater than the measured detection limit. However, the low detection

limits of the immobilized species are significant, because they allow one to characterize the species that modify the surface properties. By comparison, XPS analysis of soft-landed pure CV^+ was unable to detect the nitrogen atoms in a sample that was thoroughly washed with methanol and furnished an intense SERS spectrum, such the one shown in Figure 5.1. From the comparison of the amount of CV^+ ions that provided the Raman signal from the 4 mM solution (>50 fmol) and the estimated amount of surface-bound CV^+ ions that gave rise to the SERS spectrum in Figure 1 (<600 zeptomol), we estimate the surface enhancement factor to be on the order of 10^5, [15] which is compatible with the enhancement factors predicted by the electromagnetic theory of SERS.[16]

A significant advantage of SERS spectroscopy of soft-landed ions is that the metal surface efficiently quenches analyte fluorescence. The SERS spectrum measured with a thick layer of ca. 0.5 nmol of soft-landed Rhodamine B that was not washed shows an intense fluorescence band at 537 nm that obscures the Raman bands (Figure 5.9a). The fluorescence band completely disappears in the sample where the unbound layer of Rhodamine B was washed off leaving only the surface-bound material that shows an intense SERS spectrum (Figure 5.9b). This phenomenon is understandable given the known strong quenching effect of conductance band electrons in bulk metal[17] that decreases with the third power of distance from the surface[18] and thus mainly affects the surface-tethered species.

5.4 Surface Properties

The finding that the surface-immobilized material shows the essential features of the same species in solution raises the question of the nature of the surface and the analyte binding to it. To address this question, I first investigated the surface of the silver targets before and after treatment with air-Ar plasma and following soft landing. AFM scans of these silver targets revealed substantial differences. The untreated Ag surface shows a relatively low roughness (Figure 5.10a). The plasma-treated SERS active surfaces show a number of ellipsoid-shaped

protrusions that increase the average surface roughness to 22-24 nm, when expressed as an arithmetic average of deviations along the *z*-axis from the center plane (Figures 5.10b and c). The size of the protrusions in the *x*-*y* plane is estimated from the AFM scans to be 20-40 nm, which is in the same range as for SERS-active metal colloid particles.[10,11] However, the important practical difference from colloidal Ag is that plasma-etched Ag surfaces can be made reproducibly SERS active, and they remain stable and SERS active for the period of several weeks, even when exposed to the atmosphere.

As was already emphasized several times, surface oxidation is a necessary condition for non-destructive soft-landing and immobilization of ions on metal surfaces. SERS activity was shown previously for silver oxide layers sputtered on Al/glass substrates and exposed to photolytic reduction.[9b] and for silver atoms and ions sputtered on various metal and refractory substrates.[9a] In contrast to plasma treatment, the previous modes of activation for SERS relied on a photolytic modification of the surface by laser irradiation.[9] For plasma treated Ag surfaces, the layer of silver oxide is reduced at the ion impact site by electrons from the bulk metal that are attracted by the positive charge of the soft-landed ion. The surface-tethered permanent cations such as CV^+ and Rhodamine B probably bind by ion-pairing with oxide anions in the surface lattice. This is consistent with the SERS spectra of the soft-landed ions that show all features of Raman spectra of the corresponding ions in solution. Charge neutralization in the outer, loosely bound, layers of CV^+ probably occurs by ion pairing with hydrated OH^- ions produced by reduction of the surface silver oxide layer. This mechanism is corroborated by an experiment in which an Ag substrate was plasma-treated in O_2 (0.25 Torr, 1.5 min, 60 W) to form a layer of Ag oxides. This surface was dull black, non-conductive, and showed a different morphology than an untreated Ag surface when examined by optical microscopy. The treated surface was then exposed to the CV^+ ion beam at 50 eV and 2 nA. The substrate area that was exposed to the ion beam was found to be conductive and showed the formation of silver metal clusters that were visible by

optical microscopy. In contrast, the surface area that was not exposed to the ion beam showed no changes. This indicates that the surface oxide layer undergoes reduction to generate counterions that compensate the coulomb charge of the soft-landed cations.

5.5 Soft Landing of Nucleoside Ions

The CV^+ and Rhodamine B dyes are rugged aromatic molecules that have absorption maxima near the 514.5 nm excitation wavelength from the Ar ion laser. Soft landing of such ions may be expected to yield undamaged material on the surface. In addition, the SERS signal produced by surface-tethered CV^+ and Rhodamine B is amplified by resonant excitation of their chromophores. To explore the applicability of SERS for the characterization of more fragile biological molecules that lack chromophores for resonant excitation, I investigated soft landing of the DNA nucleobase cytosine and the nucleosides cytidine, and 2'-deoxycytidine. All these molecules produced singly protonated cations in electrospray[19] that were soft-landed on plasma-treated Ag substrates at 40 eV. The soft-landed samples of cytosine, cytidine, and 2'-deoxycytidine gave reproducible SERS spectra. The spectra were interpreted by comparing them with the experimental and calculated Raman spectra of the relevant cations and neutral molecules, as illustrated and discussed for cytidine.

The structures and relative free energies of the species involved in the soft-landing experiments are addressed first. Cytidine is calculated to form two stable conformers (**1** and **2**, Figure 5.11) that differ by the orientation of the nucleobase moiety with respect to the ribofuranose ring. Conformer **1**, in which the cytosine carbonyl is intramolecularly hydrogen bonded to O-2'—H, is calculated to be 11 kJ mol^{-1} more stable in the gas phase ($_{\Delta}G_{g,298}$ value) than **2** in which the carbonyl is hydrogen bonded to O-5'—H. The structures of cytidine conformers are affected by solvation with water. In particular, solvation of **1** disrupts the intramolecular hydrogen bond of the cytosine carbonyl (Figure 5.11) which affects the relative

thermodynamic stabilities of solvated **1** and **2** that show $_{\Delta}G_{aq,298}$ (**1** → **2**) = -0.3 kJ mol^{-1}. Gas-phase protonation of **1** is calculated to occur in the cytosine ring at N-3 producing ions **1Ha**$^+$ and **1Hb**$^+$ or at O-2 to produce **1Hc**$^+$. The corresponding gas-phase basicities were calculated as GB = 909, 924, and 903 kJ mol^{-1}, for the formation of **1Ha**$^+$, **1Hb**$^+$, and **1Hc**$^+$, respectively. Protonation at N-3 in **2** results in a disruption of the hydrogen bond to O-5'—H, and the cytosine ring rotates to achieve structures **1Ha**$^+$ and **1Hb**$^+$ as local energy minima (Figure 5.12). Solvation in water prefers **1Ha**$^+$ over **1Hb**$^+$ and **1Hc**$^+$, as illustrated by the calculated $_{\Delta}G_{rxn,298}$ for proton transfer to water (eq 5.1-5.3).

1Ha$^+$(aq) + H_2O(aq) → **1**(aq) + H_3O^+(aq) $\quad _{\Delta}G_{rxn,298}$ = 67 kJ mol^{-1} (5.1)

1Hb$^+$(aq) + H_2O(aq) → **1**(aq) + H_3O^+(aq) $\quad _{\Delta}G_{rxn,298}$ = 57 kJ mol^{-1} (5.2)

1Hc$^+$(aq) + H_2O(aq) → **1**(aq) + H_3O^+(aq) $\quad _{\Delta}G_{rxn,298}$ = 36 kJ mol^{-1} (5.3)

Hence, the thermodynamic data indicate that cytidine ions formed by electrospray are likely to have structure **1Ha**$^+$.

The SERS spectrum of soft-landed **1Ha**$^+$ (Figure 5.13) shows several bands that can be assigned on the basis of the Raman spectra that were measured in aqueous solution (Figure 5.14) and also by comparison with the previous solution Raman and SERS spectra obtained on silver colloid particles.[20] Reference Raman spectra were also calculated for cytidine molecules in aqueous solution (Figure 5.13b) and the gas phase (Figure 5.13c). Raman active vibrational modes in cytidine are listed in Table 5.1. In comparison with reference spectra, the SERS spectrum in Figure 5.13a shows substantially broadened bands in the entire wavelength region. Enhancement by SERS is observed for the bands corresponding to cytosine ring deformation modes of the $N_{(1)}$–$C_{(2)}$–$N_{(3)}$ bonds (768 cm^{-1}), the $N_{(7)}$–$C_{(4)}$ and $C_{(5)}$–$C_{(6)}$ symmetric stretch in the cytosine moiety (1616 cm^{-1}), and the ribofuranose C–H bond bending modes at 1345 and 1361 cm^{-1} that appear at 797, 1609, 1322, and 1363 cm^{-1}, respectively. In contrast, the strong Raman band of the asymmetric

$C_{(5)}$–$C_{(4)}$–$N_{(3)}$ ring stretch (calculated at 1518 cm^{-1}, measured at 1528 cm^{-1})[41] is relatively less enhanced in SERS for both soft-landed ions and cytidine absorbed on silver colloids.[41] Also noteworthy is the shoulder at 1637 cm^{-1} in the SERS spectrum that corresponds to the symmetric $C_{(5)}$–$C_{(6)}$ and $C_{(2)}$–$N_{(3)}$ combination stretch in the cytosine ring of the solvated molecule at 1636 cm^{-1} (Figure 5.13b). This band was previously assigned to the C=O stretch,[20,21] but the present vibrational analysis clearly identifies it as the above-mentioned combination mode. In contrast, the C=O stretch in gas-phase cytidine, which is calculated to be at 1690 cm^{-1}, is very weak in the SERS spectrum, which indicates that the surface-bound molecules are mostly microsolvated.[22] The SERS spectrum also differs in several wavenumbers from the calculated Raman spectra of rotamers of protonated cytidine cations (Figure 5.15). This indicates that the molecules on the surface are neutral, and hence the soft-landed cations must have been neutralized by proton transfer to the oxide layer.

The enhancement observed for the Raman bands originating from the ribofuranose bending modes and the cytosine ring-deformation modes indicates that the soft-landed cytidine molecules assume random orientation on the surface. Multiple orientations on the surface also explain the peak broadening in the SERS spectra of soft-landed cytidine, because both the intensity and wavenumbers of the Raman bands depend on the contact with the Ag surface. The finding that the soft-landed molecules and those in solution display the same vibrational modes strongly indicates that the majority of the soft-landed molecules were not damaged by the 40-50 eV impact with the surface, nor did they form covalent bonds to the oxide layer that would disrupt the weak $N_{(1)}$–$C_{(1)}'$ cytosine-sugar glycosidic bond. Protonated and neutral cytidine are calculated to have a rovibrational enthalpy of 39-40 kJ mol^{-1} at 298 K. Thus, a full conversion of a 40 eV impact energy into the internal modes of the soft-landed molecule would be equivalent to heating it to 29,500 K. The fact that the soft-landed molecules survive intact indicates that the impact energy is very rapidly dissipated to the surface. Indeed, molecular dynamics calculations of fast ions impacting on Ag surface predicted energy dissipation within 500 fs.[23] Such a

time scale is too short to allow a polyatomic molecule to dissociate. The impact energy of the soft-landing ion can be used, in part, to temporarily desorb water and methanol molecules from the impact site on the surface. However, the sample is exposed to water and methanol vapor at partial pressures (estimated at low 10^{-4} Torr) that almost guarantee resorption from the gas phase for the duration of the soft landing experiment. This explains why the soft-landed cytidine molecules appear hydrated in the SERS spectra.

The nature of bonding to the surface can be deduced only indirectly from the present data. The SERS spectra show that the soft-landed molecules do not develop covalent bonds to the surface atomic or molecular species as previously thought. It appears likely that the bonding is mediated by π-coordination of the cytosine ring to the surface silver atoms and ions.

5.6 Conclusion

In summary, polyatomic cations that are soft-landed on plasma-treated Ag surfaces at hyperthermal impact energies exhibit reproducible surface-enhanced Raman scattering effects that can be used to characterize the chemical nature of the surface-bound ions and molecules. From the present study it appears that the molecules are bound to the surface by π-coordination to the Ag atoms and ions. The discovery of reproducible SERS for soft-landed ions opens new avenues to investigate soft-landing technology for the fabrication of bioactive metal surfaces and biomolecule immobilization.

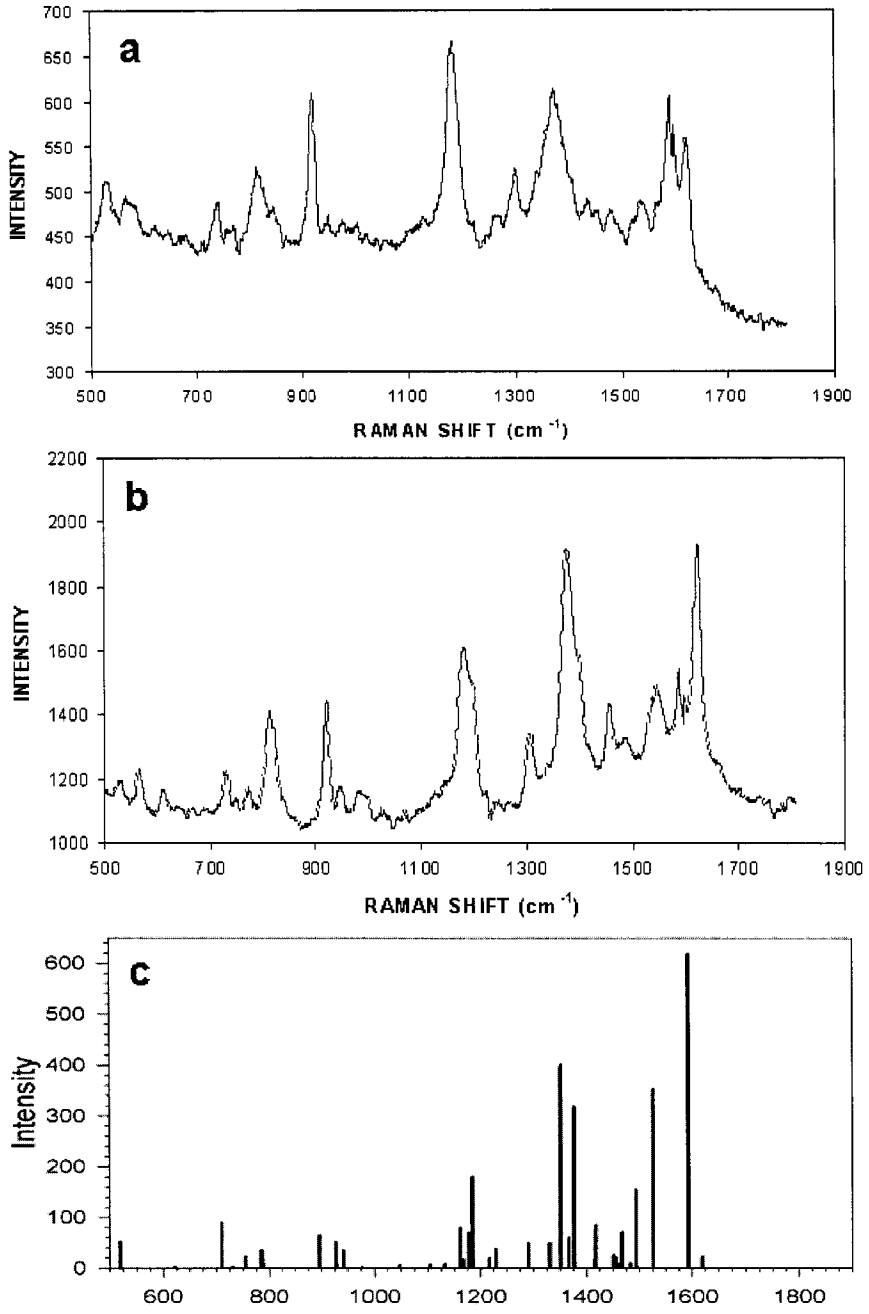

Figure 5.1 SERS spectrum of Crystal Violet.

(a) SERS spectrum of soft-landed crystal violet cation on a plasma-treated Ag substrate. (b) Raman spectrum of 4 mM solution of CV^+ chloride in methanol. (c) B3LYP/6-31G(d) calculated Raman spectrum of gas-phase CV^+. The wavenumbers in the calculated spectrum were scaled by 0.981.

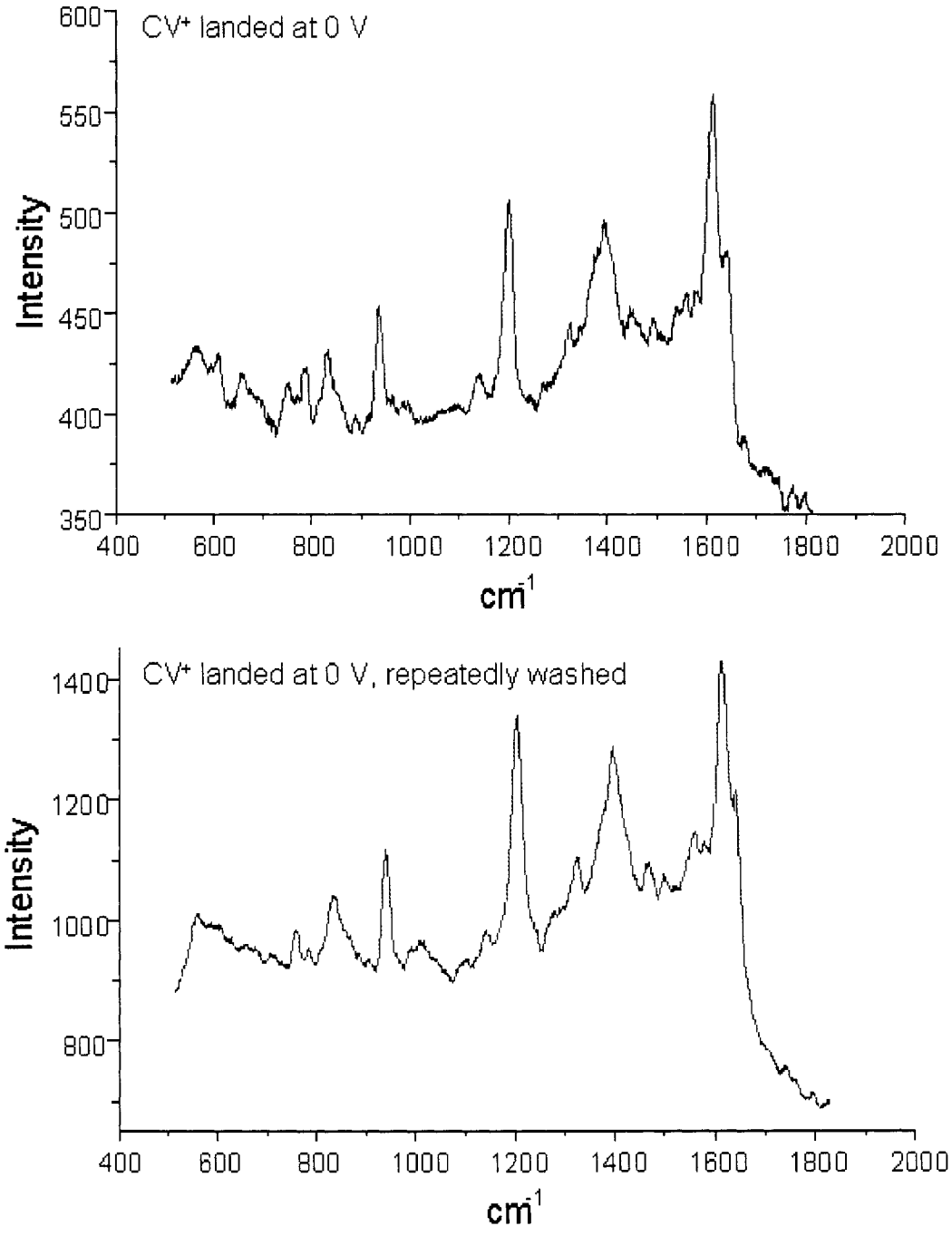

Figure 5.2 SERS spectra of crystal violet soft-landed at no acceleration.

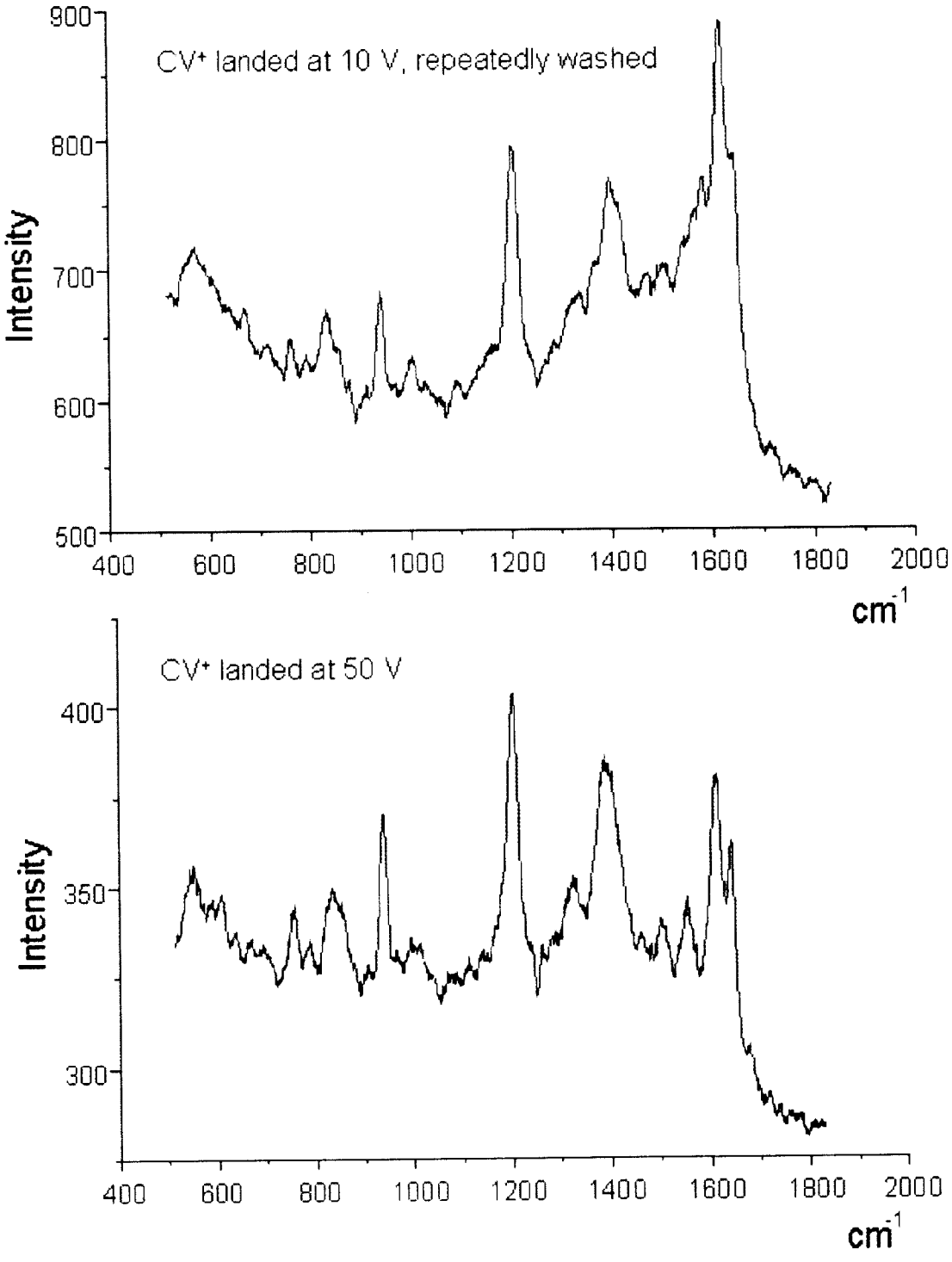

Figure 5.3 SERS spectra of crystal violet soft-landed at 10 and 50 eV.

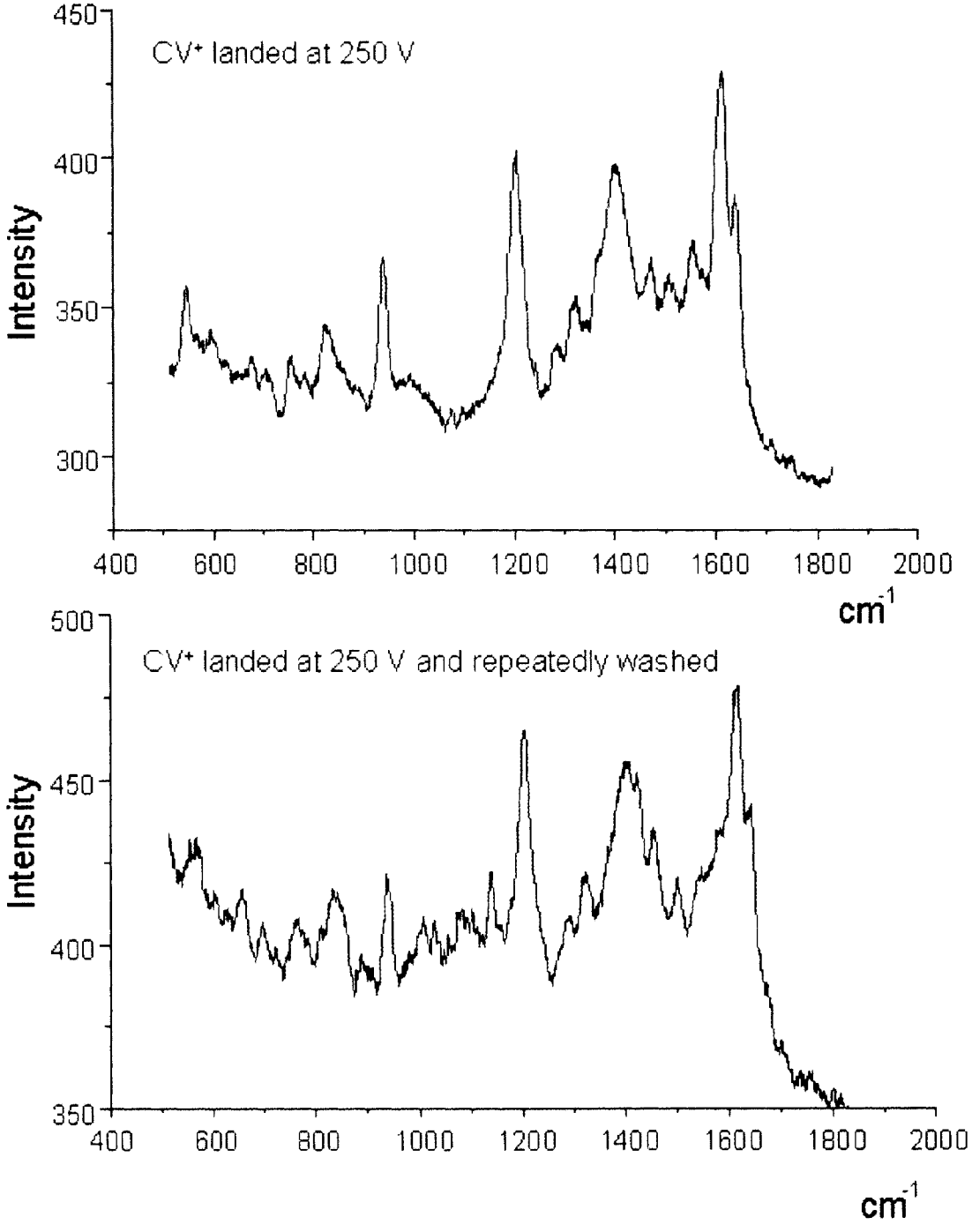

Figure 5.4 SERS spectra of crystal violet soft-landed at 250 eV.

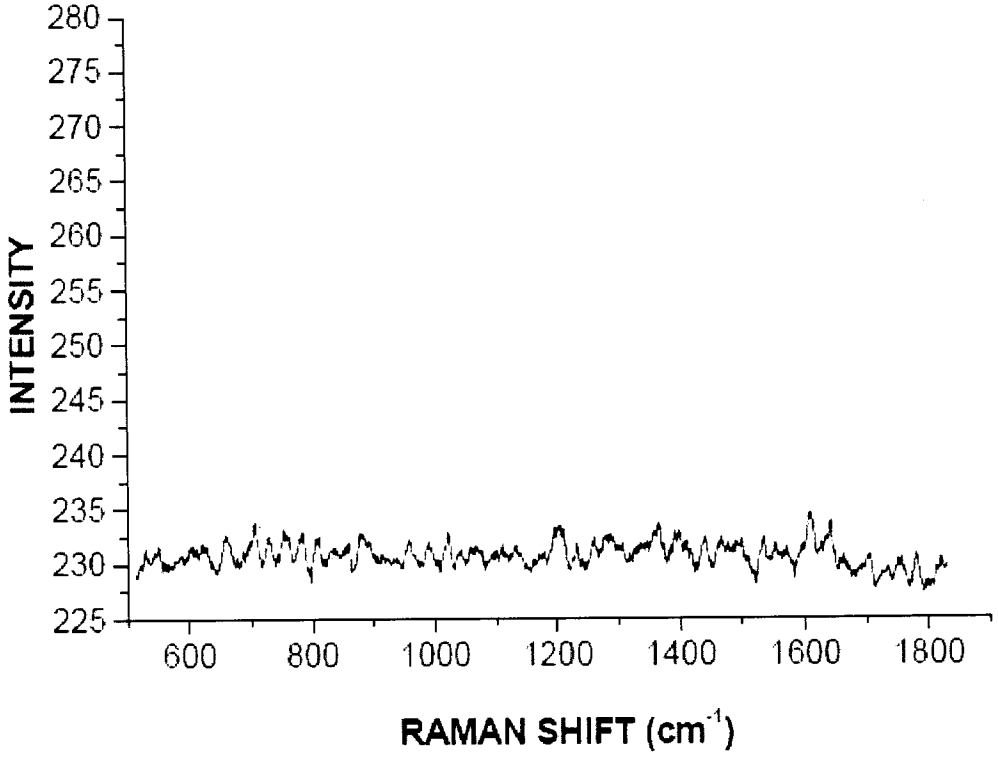

Figure 5.5 Raman spectrum of blank surface.

Raman spectrum taken from a plasma-treated Ag surface that was exposed to 3 μM CV^+ solution for 15 hrs.

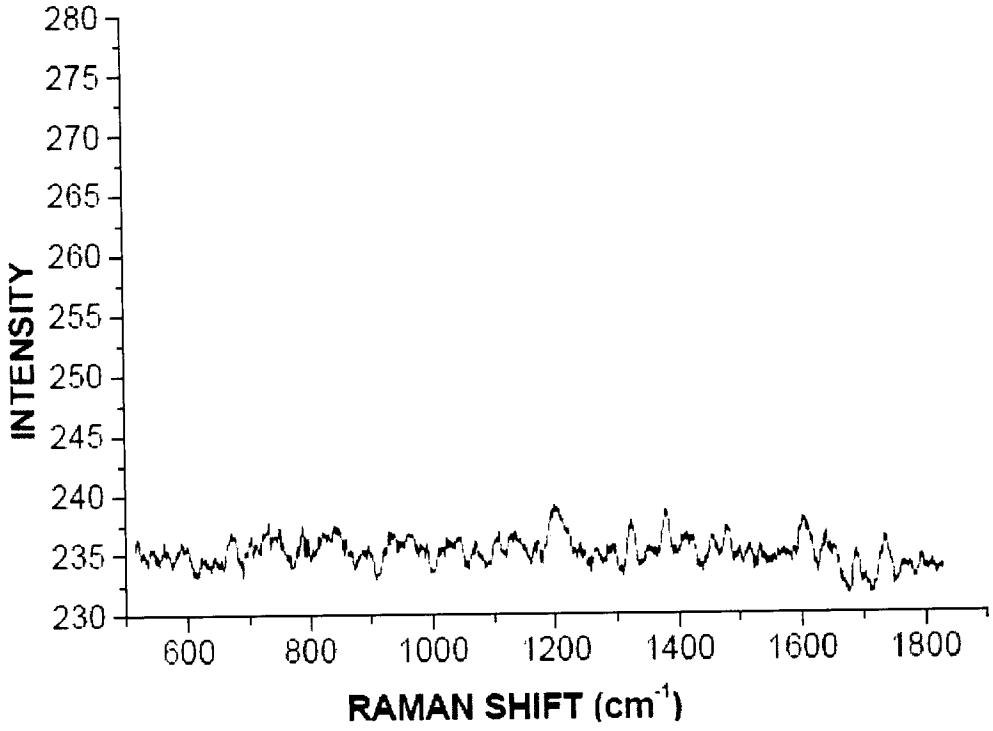

Figure 5.6 Raman spectrum of blank surface.

Raman spectrum of 2 nmol of crystal violet that was dried on a plasma-treated Ag surface.

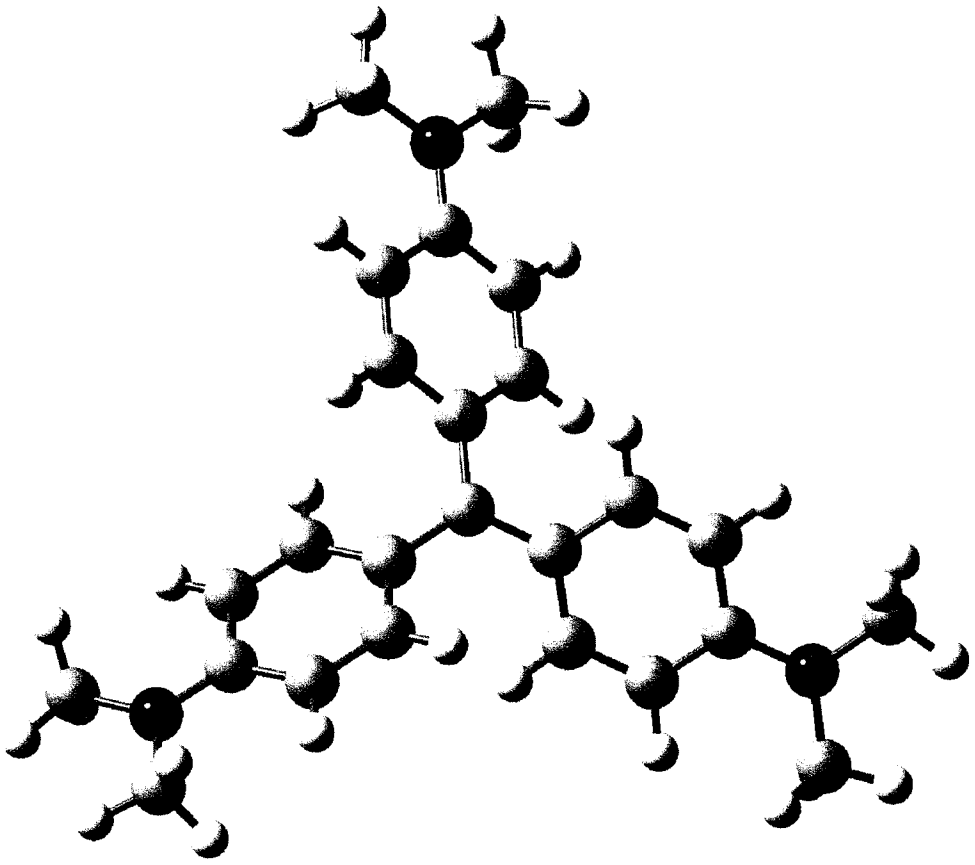

Figure 5.7 optimized structure of CV.

B3LYP/6-31G(d) optimized structures of crystal violet in the gas phase. The ion shows C_3 symmetry with the symmetry axis running through the central carbon atom. The benzene rings are twisted in a propeller-like fashion by 32.5° out of the C—C—C plane.

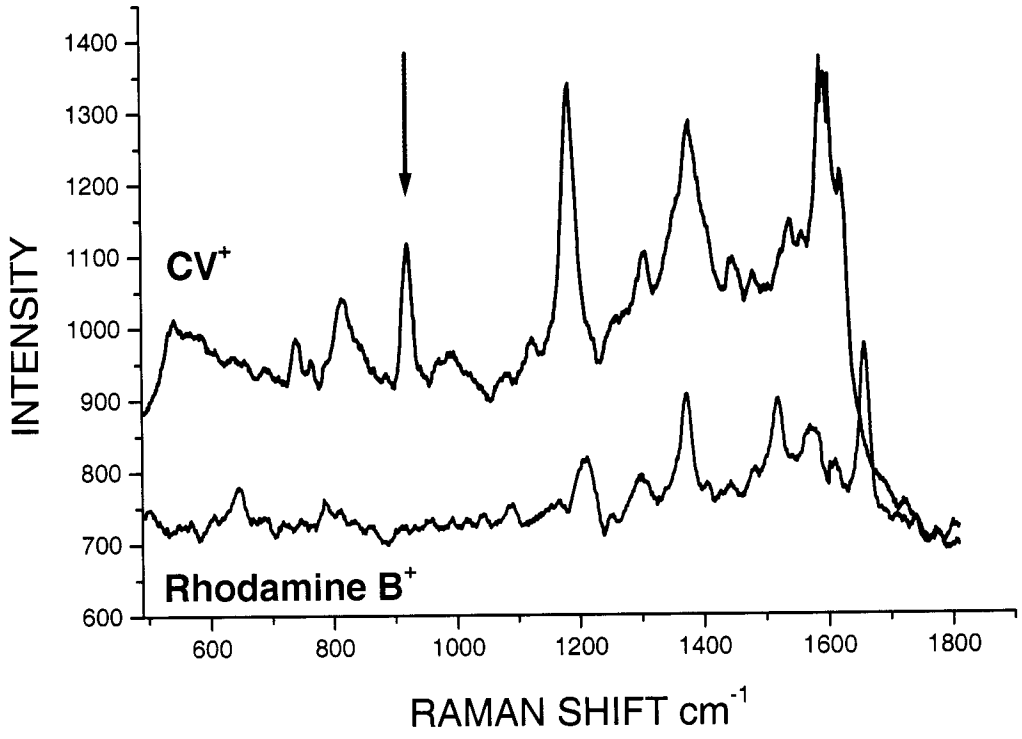

Figure 5.8 SERS spectra of soft-landed CV^+ and Rhodamine B.

SERS spectra of soft-landed CV^+ (top trace) and Rhodamine B (bottom) that were deposited at comparable concentrations. The arrow indicates the unique peak that was used for CV^+ quantification.

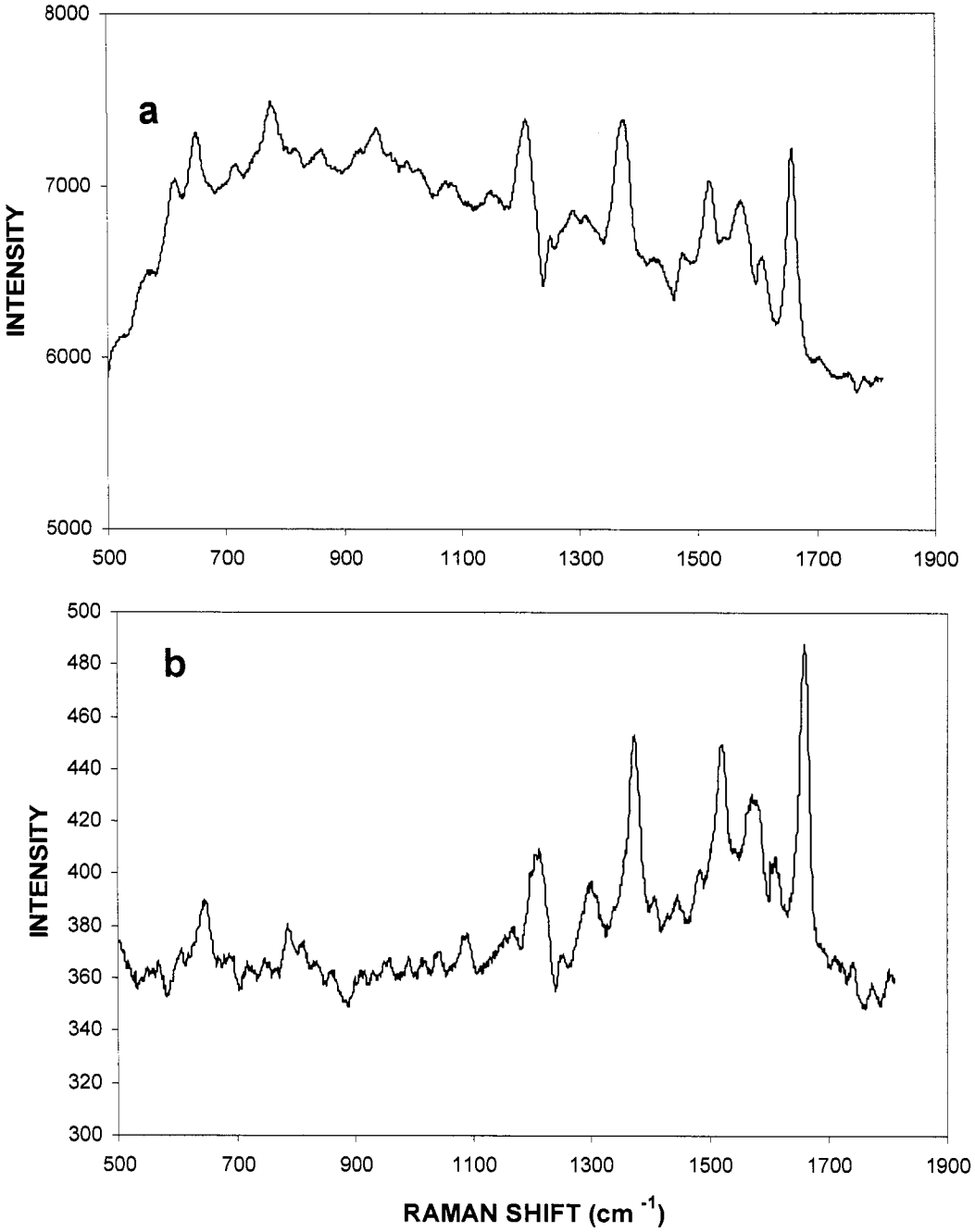

Figure 5.9 SERS spectrum of Rhodamine B.

(a) SERS spectrum of Rhodamine B that was soft-landed on the plasma-treated Ag surface without washing. (b) SERS spectrum after washing off the unbound Rhodamine B. Note the >200 fold decrease of the fluorescence background centered around 537 nm.

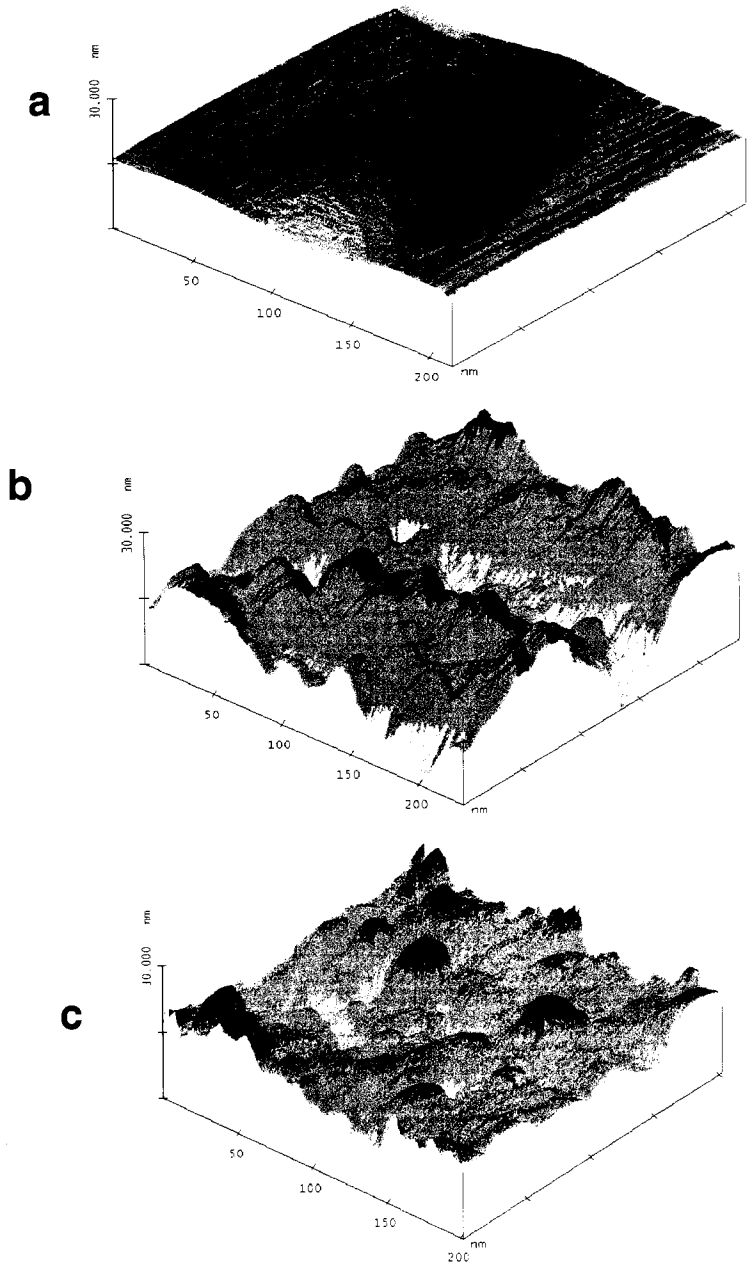

Figure 5.10 Atomic force microscopy scans of silver substrates.

Atomic force microscopy scans of 200 × 200 nm sections of silver substrates. (a) Untreated Ag substrate, (b) plasma-treated Ag substrate, and (c) plasma-treated Ag with soft-landed CV^+ ions. The size of a CV^+ ion is much smaller as approximated by its van der Waals radius (0.85 nm).

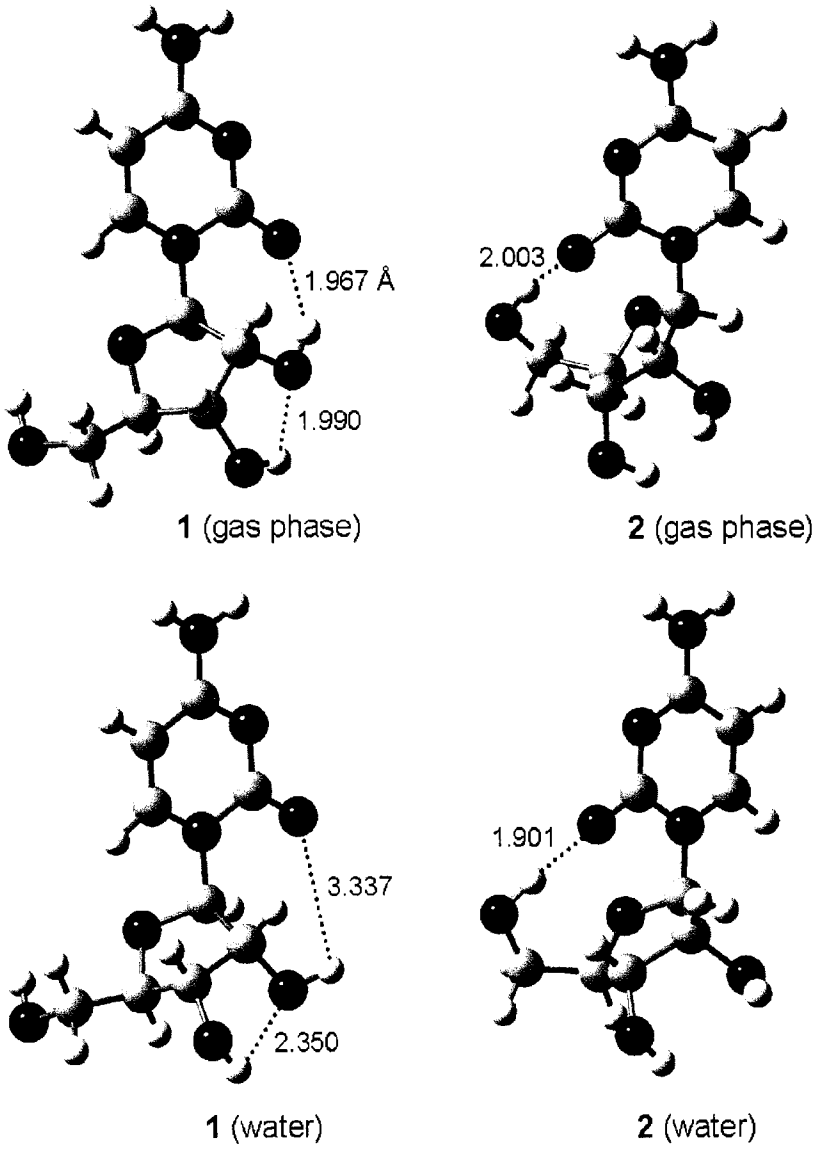

Figure 5.11 Optimized structures of cytidine conformers.

B3LYP/6-31+G(d,p) optimized structures of cytidine conformers **1** and **2** in the gas phase (top) and in the water dielectric (bottom). Broken lines indicate intramolecular hydrogen bonds in angstroms.

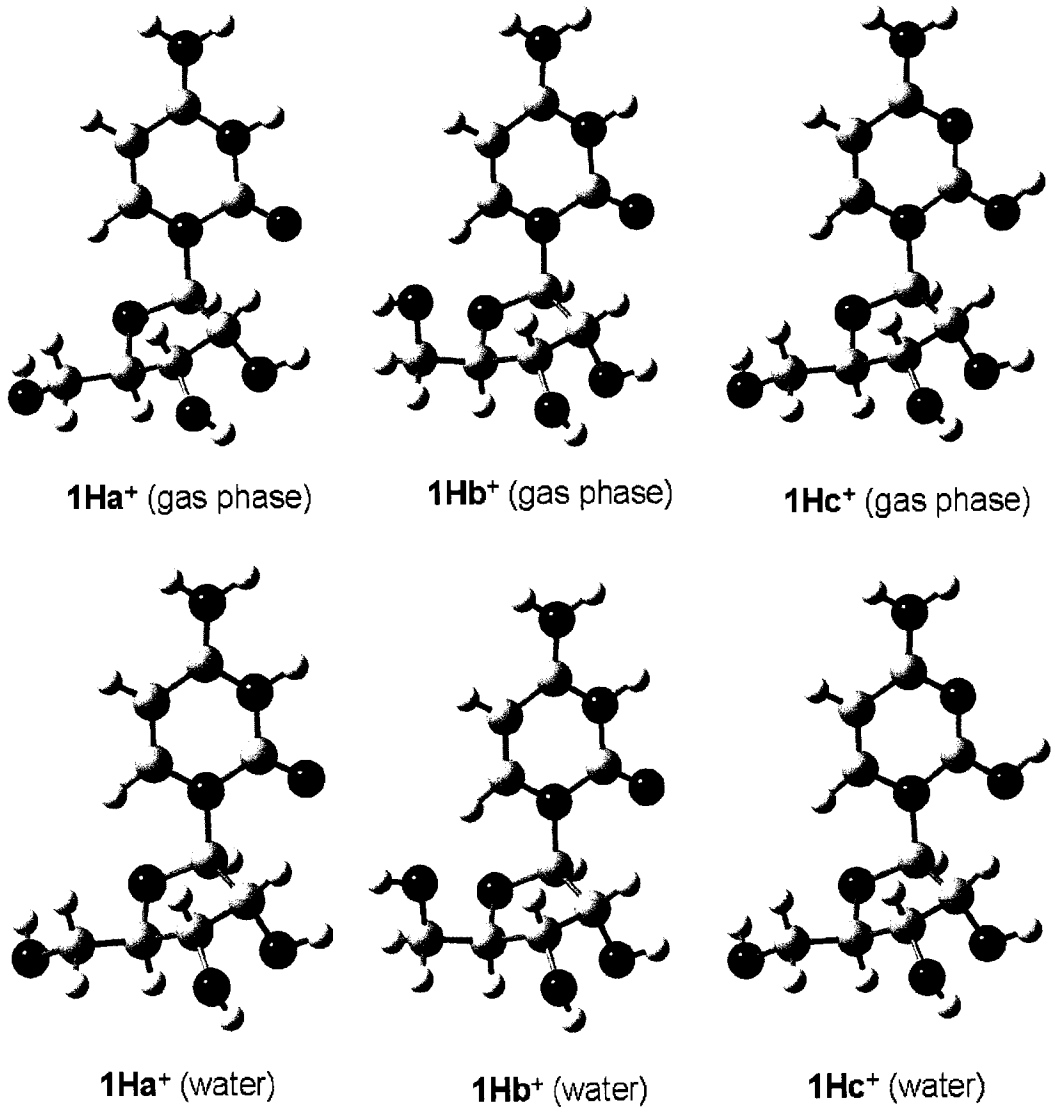

Figure 5.12 Optimized structures of conformers of protonated cytidine.

B3LYP/6-31+G(d,p) optimized structures of conformers of N-3 protonated cytidine cation **1Ha**$^+$ and **1Hb**$^+$, and the O-2 protonated tautomer **1Hc**$^+$. Top structures show gas-phase ions, bottom structures are for cations in the water dielectric.

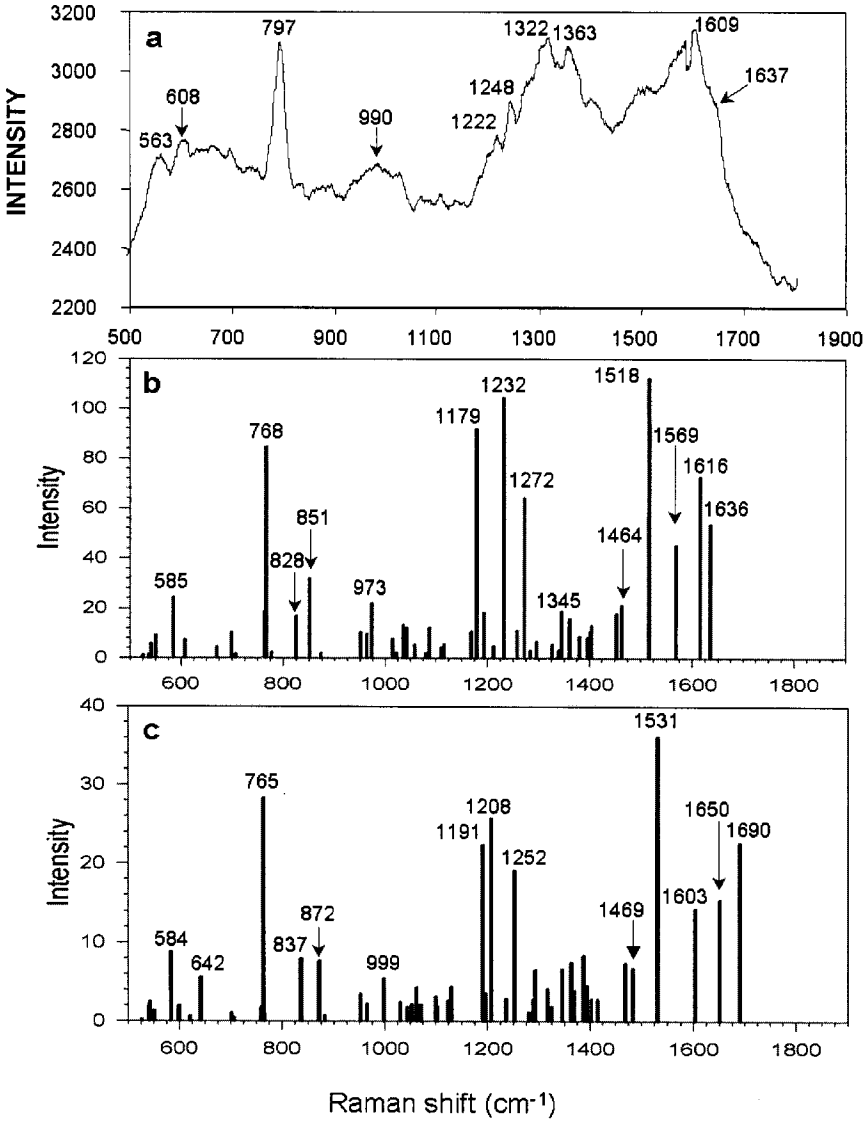

Figure 5.13 SERS spectrum of cytidine.

SERS spectrum of cytidine soft-landed at 40 eV on a plasma-treated silver substrate. (b) PCM-B3LYP/6-31+G(d,p) calculated Raman spectrum of cytidine conformer **1** in the water dielectric. (c) B3LYP/6-31+G(d,p) calculated Raman spectrum of gas-phase cytidine conformer **1**. The wavenumbers in the calculated spectra were scaled by 0.98.

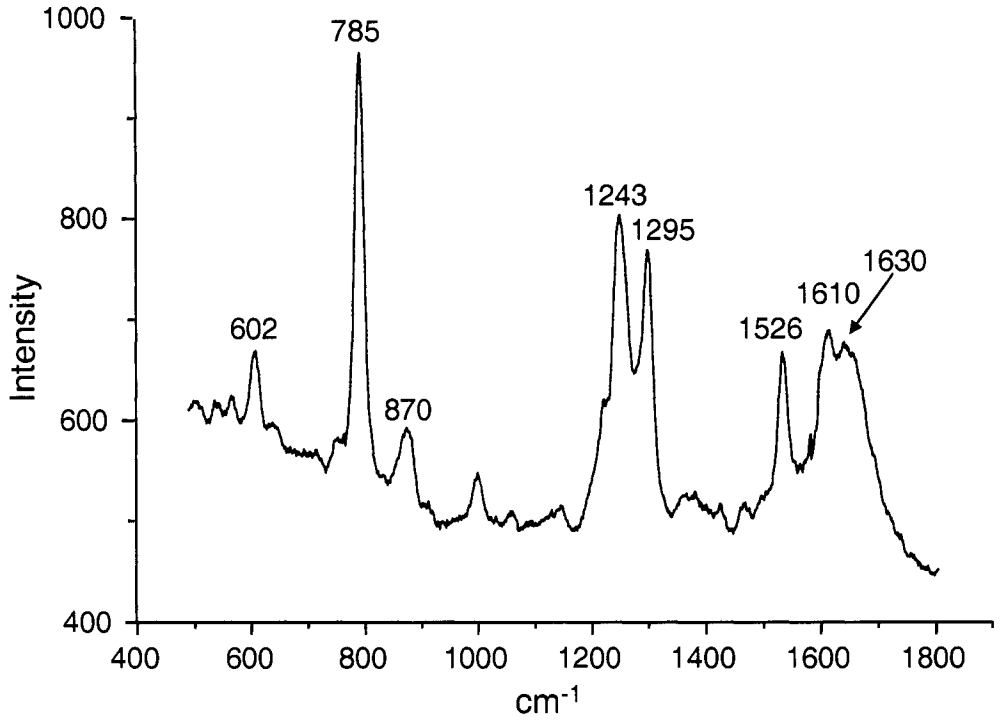

Figure 5.14 Reference Raman spectrum of cytidine solution.

Raman spectrum of cytidine solution in water at 20mg/mL (0.08M) concentration.

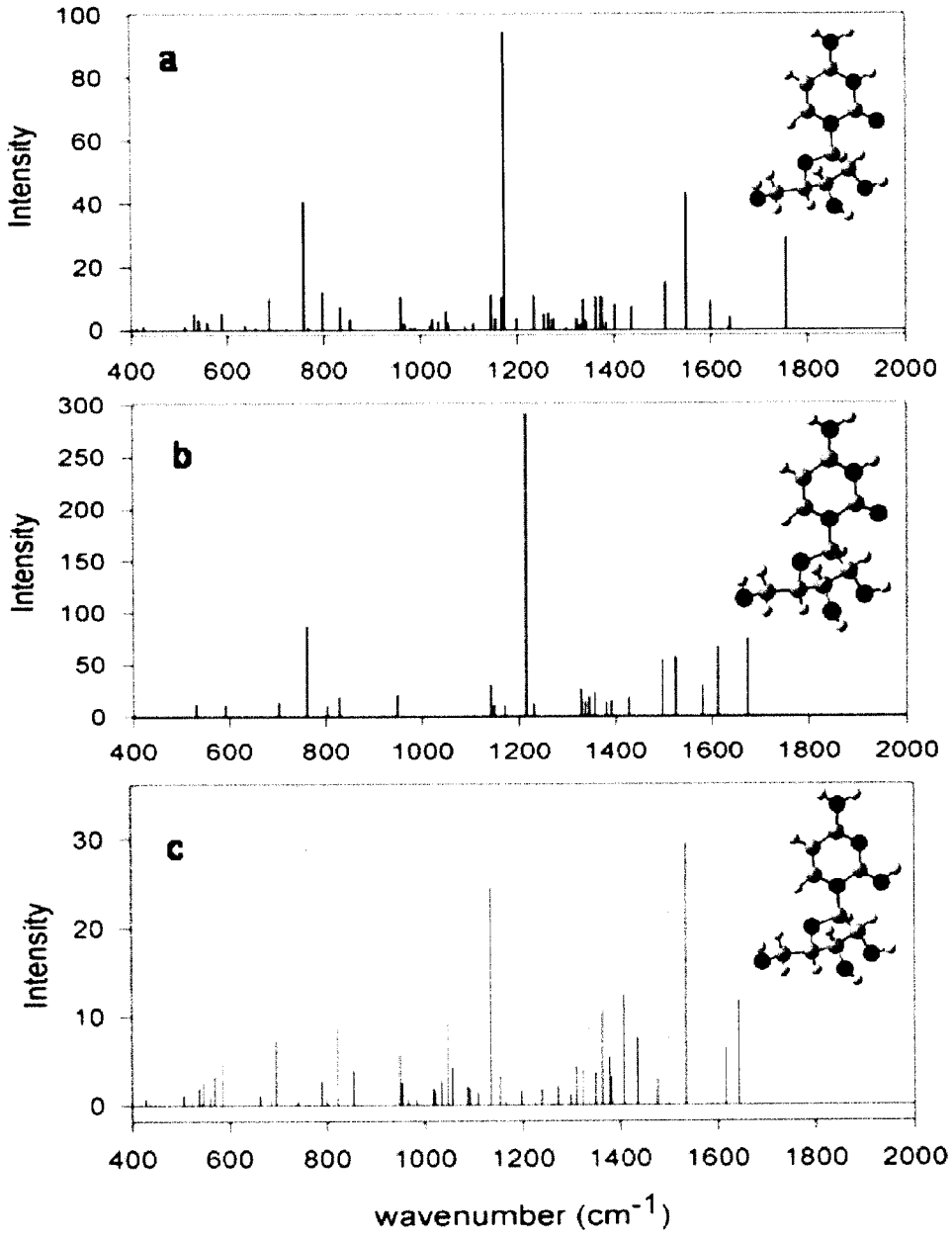

Figure 5.15 Calculated Raman spectra of rotamers of protonated cytidine cations

(a) N-3-protonated cytidine **1Ha**$^+$ in the gas phase, (b) **1Ha**$^+$ in aqueous solution, and (c) O-2-protonated cytidine **1Hc**$^+$ in the gas phase.

Table 5.1 Raman active vibrational modes in cytidine.

Wavenumber (cm^{-1})				
Gas phase		Aqueous		
ν	ν(corr)[a]	ν	ν(corr)[a]	assignment
595	584	596	585	cytosine ring deformation at $N_{(1)}$–$C_{(2)}$–$N_{(3)}$
779	765	783	768	symmetrical cytosine ring breathing
853	837	842	826	$C_{(5)}$'–$H_{(2)}$' methylene rocking
889	872	868	851	$C_{(2)}$'–$C_{(3)}$' furanose ring stretch
1214	1191	1202	1179	$N_{(1)}$–$C_{(1)}$' stretch
1231	1208	1256	1232	$H_{(2)}$'–$C_{(2)}$'–$C_{(3)}$'–$H_{(3)}$' in-plane bend
1277	1252	1297	1272	$N_{(1)}$–$C_{(2)}$–$N_{(3)}$ asymmetric ring stretch
1372	1345	1371	1345	coupled furanose C–H and O–H bends
1389	1363	1387	1361	coupled furanose C–H and O–H bends
1415	1388	1429	1402	furanose $O_{(2)}$'–H and $O_{(3)}$'–H bends
1498	1469	1482	1454	furanose $C_{(5)}$'–H_2 scissors
1513	1484	1493	1464	$N_{(1)}$–$C_{(6)}$,$N_{(3)}$–$C_{(4)}$–$N_{(7)}$asymmetric ring stretch
1561	1531	1547	1518	$N_{(3)}$–$C_{(4)}$–$C_{(5)}$ asymmetric ring stretch
1634	1603	1599	1569	H–$N_{(7)}$–H scissor
1682	1650	1668	1636	$N_{(7)}$–$C_{(4)}$, $C_{(5)}$–$C_{(6)}$ symmetric stretch
1723	1690	1647	1616	C=O stretch

[a]Scaled by 0.981.

5.7 Notes to Chapter 5

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(15) XPS is typically able to identify any element (except H and He) present at concentrations >0.1 atomic % in the outermost 10 nm of a surface (see, for example: (a) Ratner, B.; Castner, D. In *Surface analyses;* Vickerman, J.C., Ed.; Wiley: Chichester, 1997; pp 43-98. (b) Siegbahn, K. *Science* **1982**, *217*, 111-121). The fact that XPS was unable to detect nitrogen in the SERS active layer indicates that the surface packing of CV^+ molecules (containing ~5 atomic % of nitrogen) that remain on the surface after washing is significantly less dense than what would correspond to the close packing arrangement. Thus, the SERS detection limit is likely to be in the subzeptomol range, and the corresponding surface enhancement factor is probably in the high 10^5 to low 10^6 range.

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(22) After this SERS study of landed ions was submitted for publication, a reviewer pointed out that the shoulder at the high wave-number side of the 1650 cm^{-1} band in Figure 5.13a may be hiding a peak of an unsolvated C=O stretching vibration. However, the Raman spectrum of aqueous cytidine solution also shows a shoulder stretching to 1700 cm^{-1} (Figure 5.14). Thus, the shoulders are due to the limited resolution and perhaps other instrument factors of the Raman microscope rather than hidden absorption bands.

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CHAPTER 6: APPLICATIONS

6.1 Introduction

The primary subject of this thesis was to investigate mechanism and possibilities of soft and reactive landing of ions on plasma treated metal surfaces. This work is presented in chapters 3, 4 and 5. Nevertheless, due to the recurrent industrial funding obtained by my research supervisor, it was possible and desirable in the same time to look into the application aspects of the soft and reactive landing technology as well.

That is why the purpose of this chapter is to highlight other material that has been completed during my graduate school research. These projects that were focused on possible applications of soft/reactive landing technology were usually not the main topic of my work. Some of these results were not published yet and additional work is still in progress. They, however, represent a demonstration of possible practical applications of soft and reactive landing. These projects usually required instrumental modifications or a development of a specific method.

In principle there are four general areas of applications of soft and reactive landing:

1) *Modification of surfaces* with molecular ions that give the surface properties and functionalities that might be difficult to achieve by other techniques. I tried to reactively land hyaluronic acid –a biologically relevant macromolecule- that potentially provides increased biocompatibility of medical implants and other foreign objects that has to be in contact with tissues of living organisms. This application was the original motivation for building the soft and reactive landing instrument and the results of my work in this area are described in section 6.2.

2) *Preparative mass spectrometry* has a lot of potential in biomedical research. It was discussed in the Chapters 1 and 4 of this thesis. Its future extension into practical

applications now depends mainly on improvement of the absolute soft landing yields.

3) *Structural determination of landed ions* by different spectroscopic techniques or imaging of landed desolvated macromolecules by scanning microscopic techniques is another potentially useful application of soft landing. Extraction of ion from the gas phase can be used to confirm structure of the extracted ion or as a sample preparation tool for scanning macroscopy. The research oriented on utilization of soft landing as a tool for structiural determination is currently being done in laboratories of Professor Vicki H. Wysocki (University of Arizona, AZ)[1] and Professor Carol V. Robinson (Cambridge University, UK)[2].

4) *Preparation and modification of sensor arrays.* This application was first proposed by Cooks and it was already discussed in this thesis in Chapters 1 and 3. Project focused on the development of functional protein array is currently undergoing in the Turecek laboratory. The primary purpose of this project is to use reactive landing for deposition of protein array that could serve as s sensor for chemical weapons. The project is being done in cooperation with W. Tim Elam of UW Applied Physics Laboratory and it is funded by NSF and Combimatrix Corporation, WA. Current state of the results is summarized in section 6.3.

6.2. Enhanced *in-vitro* blood compatibility of 316L stainless steel surfaces by reactive landing of hyaluronan ions

6.2.1 Introduction

Blood compatibility is often referred to as haemocompatibility and is one of the key aspects of biocompatibility. If biocompatibility is in the most general sense defined as the ability of a material to perform with an appropriate response related to a specific application[4] then blood compatibility relates to the specific interactions between biomaterials and circulating blood.

Hyaluronan (an anion of hyaluronic acid or HA) is a naturally occurring biopolymer and a major component of many cell types' extracellular matrix (ECM).[5] It forms molecular chains of varying lengths made up of repeating sugar units. Hyaluronan belongs to the polysaccharide subgroup called glycosaminoglycans that all contain an aminosugar as a part of the repeat unit, which is usually a disaccharide. Figure 6.1 shows the disaccharide unit of hyaluronan. It consists of alternating D-glucuronic acid and N-acetylglucosamine, that are linked together through alternating β-1,4 and β -1,3 glycosidic bonds. Both sugars are spatially related to glucose which in the beta configuration allows all of the substituents to be in sterically favorable equatorial positions. Thus, the structure of the disaccharide is energetically stable. Each repeating unit contains a carboxylate group, four hydroxyl groups and an acetamido group. Unlike other important glycosaminoglycans (e.g. heparin, dermatan, chondroitin, and keratin) hyaluronan does not contain sulfate groups. Hyaluronan is found throughout the body from the vitreous humor of the eye to the synovial fluid of the joints.[6,7] It was first isolated from the vitreous body of the eye in 1934 by Karl Mayer.[8,9] Due to its ubiquitous occurrence in the human body[6], the biocompatibility properties of hyaluronan are suggested, and as a result there have been many attempts to apply hyaluronan in the area of biocompatible devices and implants. An extensive overview of the medical applications of hyaluronan and

its derivatives is available. [10] So far, the major application in the United States has been to provide a viscoelastic protective film in ophthalmic surgery.[6] The interest in immobilization of hyaluronan on hard stainless steel surfaces arises from the fact that most endovascular stents are stainless steel and that coated surfaces exhibit improved compatibility with blood cells.[11] The proposed mechanism suggests that hyaluronan coating inhibits the interaction of platelets with the bulk surface through interfering with the von Willebrand factor.[11] Also, in general, hydrogel surfaces show low adhesive interactions with proteins and cells, and this is often associated with blood compatibility. For medical implants and other devices in contact with blood, it is important to minimize the tendency of their surfaces to induce blood clotting; and the resultant thrombosis. Thus, the objective of this work was to employ hyaluronan immobilization to decrease platelet adhesion on 316L stainless steel surfaces. There are three common methods for immobilizing hyaluronan to produce biomedically useful coatings[6]. The material can either be covalently coupled to functional groups on the surface using "wet" organic chemistry [12-18], a photoreactive group can be attached to hyaluronan which is then photo-coupled to the surface [19-21], or it can be electrostatically immobilized to a positively charged surface.

An alternative dry process for immobilization of hyaluronan on stainless steel surfaces is presented here. This two step approach is based on (1) oxygen plasma cleaning and activation of the stainless steel surface that is (2) immediately followed by deposition of gas-phase hyaluronan anions. Immobilization is driven by the ion hyperthermal kinetic energy, and the process does not require additional solution chemistry. Preliminary results with reactive landing of hyaluronan using this instrument have been reported previously [25]. In this work I am addressing more detailed questions concerning the efficiency of coating large areas, and the stability and durability of hyaluronan coated stainless steel surfaces when exposed to polar solutions and body fluids under simulated physiological conditions.

6.2.2 Results

Electrospray ionization of hyaluronan, ion deposition on a small spot, and the newly built manipulator motion were extensively optimized prior to deposition on large areas. Following optimization, a set of large area samples (25 × 25 mm) coated with hyaluronan was prepared. Figure 6.2 shows a single spot of landed hyaluronan (2a) and the relation between the single spot area, limit switch settings and the large coated area (2b). The deposition time was 12 h for each sample chip. The highest obtained surface current was about 3 nA, which is comparable to ion currents obtained during single spot deposition without surface motion.

One sample was rinsed with the water/methanol mixture and analyzed by XPS. The results were compared to those previously obtained on single spot areas.[25] For the large-area XPS scan obtained at low energy resolution, the comparison focused on three elements present in HA, i.e., carbon, oxygen, and nitrogen. Table 6.1 shows that the relative abundance of C, O, and N in the single spot area and at the large are samples are in good agreement, if the usual error of XPS measurements (10% relative) is taken into account. The high resolution XPS spectra of carbon 1s in hyaluronan should contain four characteristic peaks, one for each kind of carbon that is present. Again, Table 6.1 reports very good agreement between results obtained on large areas and on single spot areas.

To test for stability of the reactively landed material, the other large area samples were exposed to the following solvents/solutions for the given time periods: deionized water (5 min), methanol (5 min), 1% sodium chloride solution (30 min), 7mM urea solution (30 min) and bovine serum (30 min). In addition to large area samples, one single spot sample was prepared as well and washed with water for 30 minutes. After exposure to solvents/solutions all samples were rinsed with deionized water and submitted to XPS analyses. The XPS results are summarized in Table 6.2.

Selected samples were submitted for platelet adhesion studies that were run at Genzyme research facilities using standard blood-materials interaction tests[26] based

on cell adhesion from platelet rich plasma (PRP). The test results are presented as scanning electron microscopy (SEM) images that allow for comparison of platelet adhesion on different sample surfaces. Figure 6.3 shows activated platelets on the plasma treated blank surface (no hyaluronan deposited) at different SEM magnifications. The blank sample clearly shows dense coverage of the surface with the platelets. In contrast, Figure 6.4 shows the results of the platelet adhesion test on a hyaluronan-coated surface that had been washed with urea solution prior to the test. The results of the test show no activated platelets on the surface of this sample. Figure 6.5 compares platelet adhesion inside the spot coated with reactively landed hyaluronan (Figure 6.5, Panel A) and outside this spot on the surrounding blank surface (Figure 6.5, Panel B). While the uncoated outside area shows abundant platelet adhesion, there are almost no platelets inside the hyaluronan deposited area.

6.2.3 Discussion

Hyaluronan anions that have been electrosprayed into vacuum and reactively landed at hyperthermal energies on plasma-treated metallic surfaces retain their biological activity. This opens several questions of charge neutralization, bond formation, and structure retention upon the ion impact on the plasma-treated surface. It was previously shown[25] that ions must be efficiently discharged upon landing to allow continuous landing of further incoming ions. Even a monolayer of surface charges at a density of one elementary charge per 50 Å would produce an electrostatic field on the order of 10^8 V/m that would be impenetrable to gas-phase ions landing at a 15 V potential applied to the landing plate. The same work also reported SEM images of landed multiple layers of hyaluronan on the stainless steel surface. As expected, the images show typical three dimensional entangled structures of polysaccharide chains and indicate that HA is deposited in several compact layers on the surface. [25] This is possible only if the charge brought in by HA anions is efficiently neutralized by a non-destructive mechanism. The plasma oxidized surface

of the landing plate consists mainly of Fe_2O_3 (and possibly Fe_3O_4 as well) and Cr_2O_3 [23], which are amphoteric oxides. Thus, charge transport can be established on the surface by protonation or deprotonation of the surface hydroxyl groups. The source of protons and hydroxyl groups on the surface is water, which is a major vacuum contaminant at the pressures used in reactive landing ($\sim 10^{-3}$ Torr). An equilibrium on the surface corresponds to an acid-base exchange that can be represented by the following dissociation reactions (≡ denotes surface groups)

$$\equiv FeOH_2^+ + B \leftrightarrow \equiv FeOH + HB^+$$
$$\equiv FeOH + B \leftrightarrow \equiv FeO^- + BH^+$$

Protonation of iron oxides is known to be exothermic with reported ΔH ranging between -25 and -48.8 kJ mol^{-1} for different oxide structures. [30,31] For a review of the kinetics, thermodynamics and mechanism of the acid-basic reactions on the surface of iron oxides see reference 32. The acidity of carboxylic groups in hyaluronan has been estimated as pK_a = 3-4 [33], so that the conjugate base has a pK_b = 10-11. This implies that carboxylate groups in hyaluronan anions are by far the strongest Brønsted bases present on the oxide surface and they can get protonated with the surface iron hydroxide groups:

$$\equiv FeOH + (OOC\text{-}Hyal)^- \rightarrow \equiv FeO^- + (HOOC\text{-}Hyal)$$
$$\equiv FeOOH + (OOC\text{-}Hyal)^- \rightarrow \equiv FeOO^- + (HOOC\text{-}Hyal)$$

Because the ion current measured on the surface is practically independent of the landing potential[22], the discharge process must also be independent of the landing energy. However, the ion kinetic energy is important for further reactions on the surface[22,23,25] because immobilization of the reactively landed material shows a threshold with respect to the ion acceleration potential. The nature of the kinetic-

energy induced surface reaction is currently under study. The process of hyaluronan immobilization on the surface is coupled with a redox reaction that is compensated by electron transfer between the oxide surface and the conduction band of the bulk metal. This electron current, as measured with a nanoamperemeter connected to the metal collector plate, is typically on the order of 10^{-9} A, which is consistent with the amount of landed material according to Faraday's law[22,23]. Thus, continuous electron transfer from the surface to the bulk metal drains the negative charge that is brought to the oxide surface by landing hyaluronan anions from the gas phase.

As mentioned above, while multiple layers of landed material are deposited in a typical landing process, only the first one or two layers can interact directly with the surface and form bonds that allow them to survive extended washing by polar solvents and solutions. The additional layers land onto the previously deposited material and are only soft landed (loosely deposited) on the surface. As a result, the loosely bound layers can be washed from the surface and recovered intact into solution, as shown previously. [22,23] Thus, the demonstrated extended blood compatibility of HA-treated stainless steel clearly results from surface protection that is provided by a thin layer, possibly even a monolayer, of hyaluronan.

In solution, the hyaluronan chains form random coils that interact with each other and cause gelation at ~1% concentrations.[6] The helical configuration of the hyaluronan polymer is due to hydrogen bonding between hydroxyl groups in the main chain and is also responsible for the overall hydrophilicity of the polymer. The random coil formed by a hyaluronan polymer is able to trap 1000 times its weight in water. [9,34] Although surface immobilization reduces the degrees of freedom in comparison with free molecules in solution, the parts of a hyaluronan chain that are facing away from the surface can be expected to interact with solvent. We envision that hyaluronan ions, that were originally desolvated by electrospray ionization at high temperature, quickly resorb water once they are discharged and cooled on the surface. Water resorption starts inside the instrument (at 10^{-3} torr, if water is the

primary species in the gas phase, a water monolayer could form in 10^{-3} sec) and it is completed once the surface is brought to ambient air conditions and exposed to wash solutions. As a result of water sorption, the immobilized hyaluronan molecules restore whole or at least significant parts of their natural solvated structure and form an entangled protective layer analogous to the protective layers previously described by Burns.[11,17] This layer swells due to water sorption and forms large domains with the actual mass of hyaluronan within domains being very low. The domains of individual hyaluronan molecules overlap each other. [33] Small molecules such as water or electrolytes can freely diffuse through the layer but large molecules such as proteins are excluded because of their hydrodynamic size in solution.

6.2.4 Conclusions

The new results reported here show that it is possible to immobilize gas-phase hyaluronan anions on large-area plasma-treated stainless steel surfaces through a dry process referred to as reactive landing. The automatic mechanical motion of the target surface is independent of the ion discharge or the immobilization process. The elemental composition of the hyaluronan-modified large-area surfaces is identical to that reported previously for single-spot modified surfaces. The immobilized hyaluronan survives extensive washing in polar solvents and solutions, and the washed surfaces maintain protective properties against blood platelet activation. The new dry *in-situ* process has the potential to improve blood compatibility of stainless steel surfaces in medical implants.

6.3 Reactive Landing of Biologically Active Proteins on a Complementary Metal Oxide Semiconductor (CMOS) Microarray

6.3.1 Introduction

The purpose of this work was to determine whether reactive landing of proteins on metal surfaces can be used to deposit biologically active enzymes on a CombiMatrix CMOS microarray. [35]

The CombiMatrix CMOS microarray (Figure 6.6 and 6.7) has 12,000 individually addressable electrodes (90, 000 arrays are currently being introduced) that can be used to monitor chemical reactions above the electrode that generate a change in current or potential (SEM of the individual electrode is shown on Figure 6.8). Because these electrical measurements are extremely small, it is critical that the CMOS circuitry remain intact and functional during the plasma process and reactive landing.

CombiMatrix claims that -if certain conditions are met- their established technology represents extremely sensitive tool for electrochemical detection of processes that take place on the surface of the array. That is why it was suggested by Combimatrix Homeland Security Division that active protein organophosphorus acid (OPA) anhydrolase could be immobilized on the electrodes of the array and its enzyme activity towards organophosphate nerve agents monitored electrochemically. This activity could be then quantified by already available technology and thus the whole device could be used as a very sensitive detector for purposes of chemical defense. The idea is schematically described on Figure 6.9.

Diisopropyl-fluorophosphatase (DFPase), Phosphotriesterase (PTE) or Organophosphorus acid (OPA) anhydrolase (EC# 3.1.8.2, CAS# 9032-18-2) is an interesting enzyme that catalyzes the hydrolysis of organophosphate nerve agents, including the chemical warfare agents VX, soman, and sarin as well as the

insecticide paraoxon. It exists (in nature) as a homodimer with one active site per monomer (first discribed in *Alteromonas sp.*). The natural substrate is not known.

The reaction was first described already in 1946.[36] Later the pure enzyme was isolated (aprox. 1980) and finally cloned in 1996.[37] Thus, the enzyme is now available by recombinant techniques from *E.coli* in relatively large preparative quantities). The recombinant enzyme functions as a 55-60 kDa monomer (sometimes it is reported as mixture of three enzymes with molecular mass ranging from 20-70 000). The exact reaction mechanism is currently being investigated[38] by U.S. Army Edgewood Chemical Biological Center. The idea to use this enzymatic reaction for biosensors is not a new one [39] but immobilization approach that utilizes reactive landing is principally novel.

6.3.2 Preliminary experiments

The first phase of this study involved repeated experiments that successfully demonstrated the deposition of streptavidin on the CMOS microarray. In these early experiments, non-functional microarrays were used and their electronics could not be mapped before and after treatment.

It was confirmed that fluorescence labeled streptavidin retains its fluorescence tag because the landed spot was detectable by fluorescence scan (Figure 6.10). In the second step the chip was exposed to the solution of biotin-cy5 conjugate, which served as a secondary fluorescence label with different emission wavelength. Again, the fluorescence was confirmed only from the area that was exposed to landing of streptavidin ions. This means that reactively landed streptavidin was able to retain its specific bioafinity towards biotin (Figure 6.11).

In the second phase the functional chips were used for the exactly same set of experiments. It was necessary to construct a special chip holder that is capable to keep the electrode array on the landing potential which is necessary to achieve immobilization. The results were same as results achieved with non-functional chips.

In addition, it was confirmed by the quality control test done in Combimatrix that chips can survive reactive landing process without any damage and the individual electrodes of the chip coated with biologically active streptavidin are still individually addressable.

The company suggested an additional test during which a compound of medium molecular mass was reactively landed on the functional chip. The chip with the landed compound was then washed (to remove the loosely bound layers) and the remaining layer was used as substrate for chemical reaction that emits fluorescence signal. The structure of the compound and the nature of the chemical reaction were not revealed by Combimatrix. However, as can be seen on Figure 6.12 and on Figure 6.13 in more details, the reaction apparently takes place only on the electrodes modified by reactive landing. On more detailed scan (Figure 6.13) even the individual electrodes can be recognized, which confirms successful reactive landing on the electrode area only.

To establish a proof of principle that enzymes can be directly applied onto the microarray and maintain their biological activity, the current effort is on the deposition of horseradish peroxidase and organophosphorus acid (OPA) anhydrolase on the array and demonstration of their biological activity by suitable enzyme assays. This research is currently being done in the W.T. Elam and F. Turecek research groups.

6.4 Reactive landing of Zirconium propoxide

6.4.1 Introduction

Zirconium dioxide (zirconia, zirconium oxide, ZrO_2) is a very suitable material for different applications. Its hardness, optical transparency and high refractive index are the most characteristic properties. [40] It is known to be one of the most stable materials both chemically and photochemically. It has excellent

mechanical, electrical, thermal and optical properties, which makes it an ideal medium for photonics applications. Zirconium dioxide has been used as an interferometry filter and for coating high power laser mirrors. [41] Thermoluminescence (TL) properties were also reported. [42,43]

Zirconia coating was reported to have several other applications. For instance, zirconia films produced through the sol-gel process [44] are known as effective in retarding corrosion of stainless steel in salt and sulphuric acid solutions [45-49], as well as in reducing high temperature oxidation of stainless steel. [45,50]

For a thin film coating such as a sol-gel-derived ceramic to remain functional it must stay well adhered throughout whatever loading the substrate may experience while in service.[51] Zirconium dioxide has a number of superior properties, such as chemical durability, alkali resistance, heat resistance against oxidation, and high hardness and refractoriness. There are several reports concerning the study of the silica-based coating techniques [6] and zirconia coatings on glass [52]. There are few reports, however, concerning zirconia coatings on stainless steel sheets.

Although the actual clinical use of zirconia is limited (mainly to use as ball heads for total hip replacement) recent studies have demonstrated the possibility of using zirconium oxide films as an articulating surface for hemiartoplasty devices.[53] However, fabrication techniques and materials choices are still a problem, especially for prostheses having complex geometry (knee etc.). In this case, the fabrication of bulk ceramic prostheses is difficult because of the high brittleness of the material itself. The possibility of developing combined metal ceramic components is believed to lead to important benefits in the implant functionality and stability. [54]

6.4.2 Deposition of zirconium dioxide by reactive landing

It is impossible to electrospray zirconium dioxide directly because it is insoluble. However, it is known that unstable organozirconium compounds can readily undergo decomposition or hydrolysis that leads to stable zirconium dioxide as a product.

Zirconium propoxide (Figure 6.14) in glass pores can be converted into zirconium dioxide by drying at room temperature and than heating at 500°C [55]. It has been reported that zirconium propoxide can be commonly used as a precursor for the preparation of zirconia[56].

For reactive landing experiment, soluble organozirconium compound (70% zirconium propoxide in 1-propanol) was further dissolved in compatible solvent (1-propanol) to the final concentration 10^{-5} M. The solution was electrosprayed and landed on plasma treated stainless steel surface. The landing experiment lasted for 3 hours and the landing energy was 50eV.

The soft landed material formed visible and very compact spot that was practically permanent. It survived long washing times (water, methanol, ethanol, propanol, hexane) for up to 3hrs and even rubbing with wet towel or filter paper. The spot did not change during washing procedures. That strongly suggests that the reaction is completed before the washing procedures were performed, probably still inside the instrument, and there is no significant amount of the zirconium propoxide left on the surface (it would undergo quick hydrolyses if exposed to washing). All samples that were prepared survived repeated washing and did not visibly change in time.

Set of samples was prepared and two were washed and examined by XPS. XPS composition scan (Table 6.3) confirmed that the spot of landed material contains zirconium. XPS detects much better surface coverage than was previously achieved on this project with other reactively landed compounds (including the original hyaluronan coating). The contamination at 285eV peak corresponds to C-C bound carbon. It can be a result of propoxide decomposition but since it normally exists on blank surfaces as well it is probable that it also originates from pump oils and other surface contaminations. The stainless steel (Fe, Cr) is very well covered under the coating and it is out of the reach of the normal XPS sampling depth.

Although more tests are needed it is reasonable to conclude that upon landing on plasma treated stainless steel chip the accelerated ion reacts with the surface water on the target chip. (water is a major vacuum contaminant at the pressure) and undergoes decomposition to zirconium dioxide (zirconia).

It was also tested if the unique coating is due to the reactive landing or if the plasma treated surface exhibits reactivity itself.

First, if the Zr-propoxide is just dried on the plasma treated surface it is easily washable and XPS detects only stainless steel and no Zr after washing (see Table 6.3).

The solution of zirconium propoxide was also electrosprayed on a plasma treated surface at ambient conditions. The electrospray needle was kept on a high potential (3.5kV) and the target plasma treated surface was grounded (similar deposition by electrospray at thermal energy conditions was reported by Morozov[57]). The immobilization was not achieved during this experiment. The electrosprayed solution did not stay on the grounded surface and was falling down in drops due to the gravity force. Thus, electrospraying at ambient conditions did not resulted in any spot of landed material on the target surface.

These control experiments are proof that reactive landing is the necessary process that achieves described surface chemistry and provides the coating with zirconium dioxide.

Tests are currently being undergone in Turecek group to find out if the zirconium dioxide prepared by reactive landing technique exhibits selective affinity towards phosphopeptides as reported for some transition metal oxides.[58]

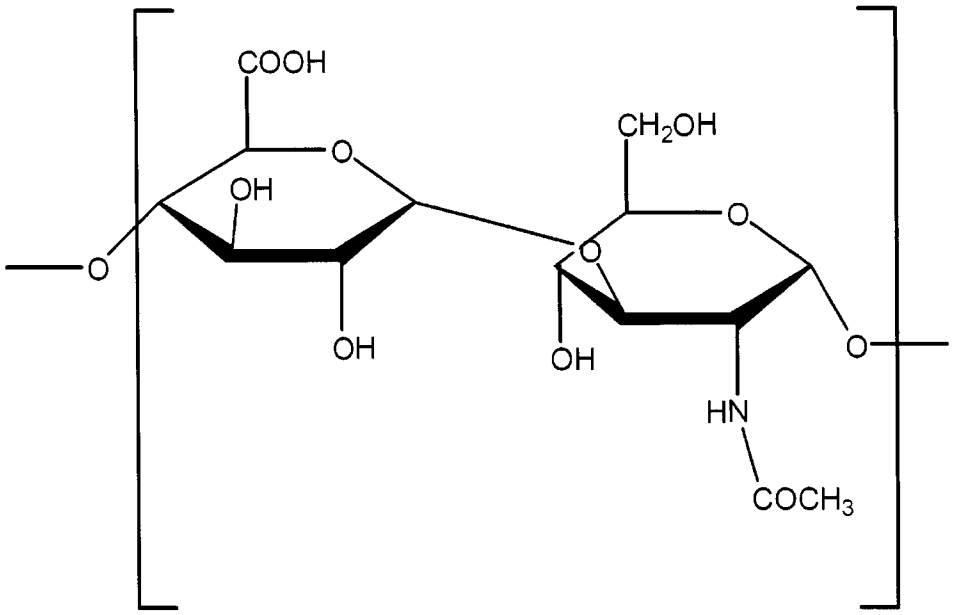


Figure 6.1 Hyaluronan structure.

Disaccharide structure of hyaluronan. This disaccharide unit is repeated (250-25000 times) resulting in hyaluronan chains with molecular mass up to several millions Daltons.

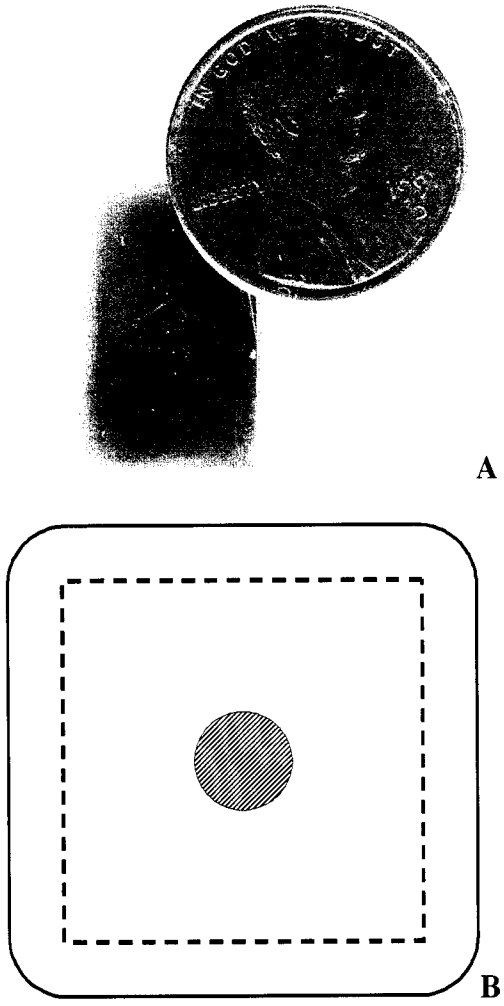


Figure 6.2 Landing area.

Panel A: Digital camera image of a landed hyaluronan spot on a plasma treated stainless steel surface. This spot represents a projection of an image of the electrostatic ion optics. Panel B: The relation between the central spot, limiting switches setup (-----) and total covered area (——).

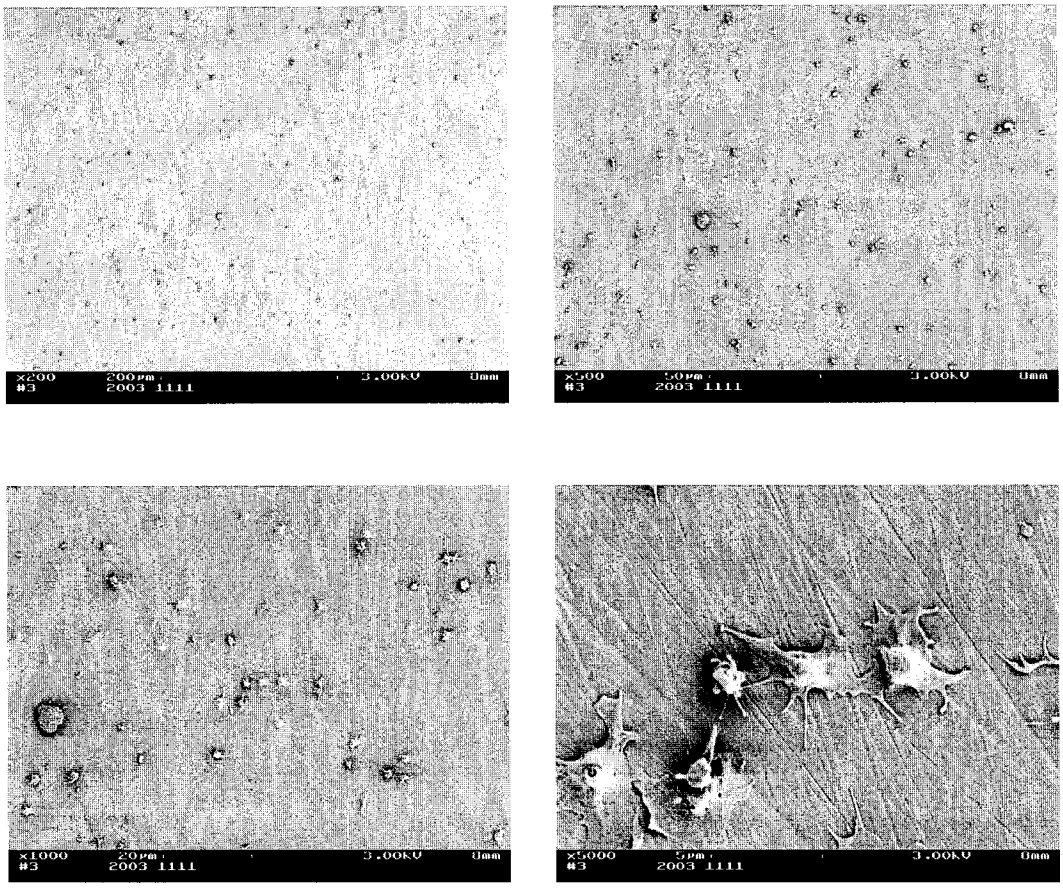


Figure 6.3 SEM images of activated platelets.

SEM images of activated platelets on a blank plasma treated surface that was exposed to the platelets adhesion study. Magnification: 200X (Panel A), 500X (Panel B), 2000X (Panel C) and 5000X (Panel D).

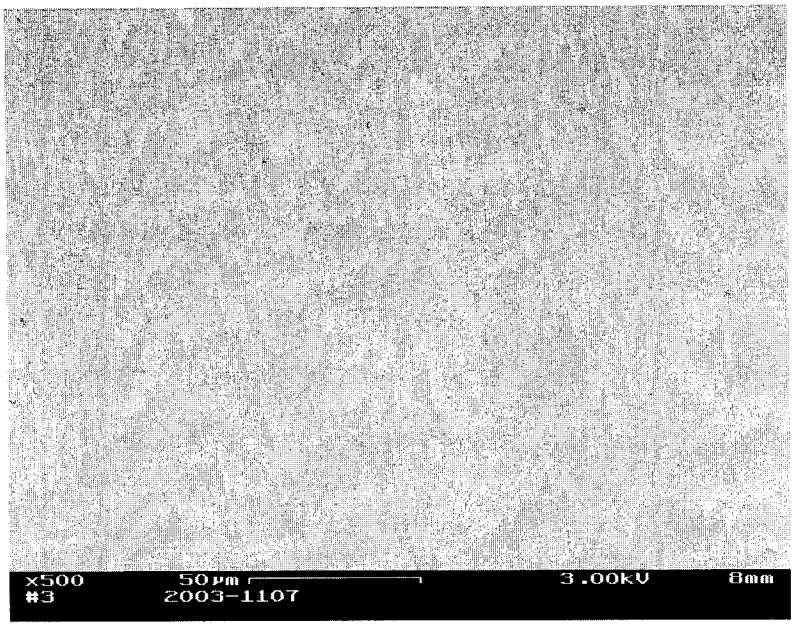
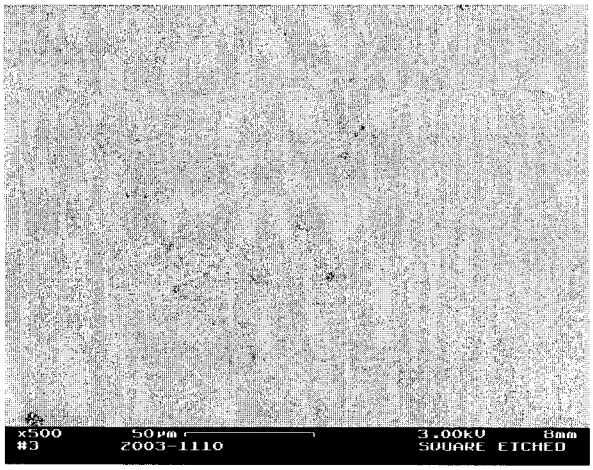
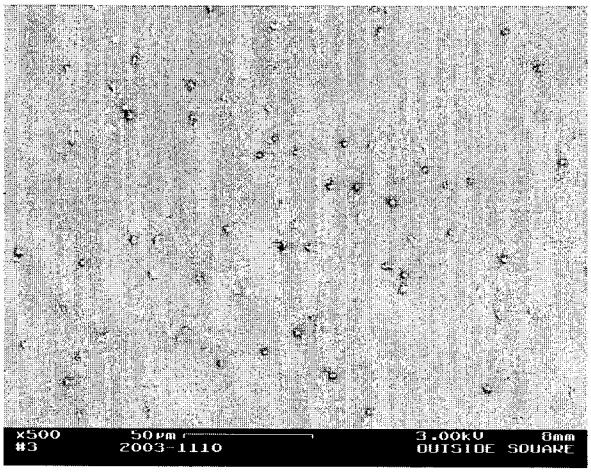


Figure 6.4 SEM image of protected surface.

SEM of a hyaluronan covered plasma treated surface that was washed by urea solution and exposed to the platelet adhesion study. No activated platelets are visible.



A



B

Figure 6.5 SEM images of activated platelets and protected surface.

SEM of the sample surface that was washed and exposed to the platelet adhesion study. Panel A: inside the spot of deposited hyaluronan, Panel B: outside of the hyaluronan spot on the blank surface (no hyaluronan deposited).

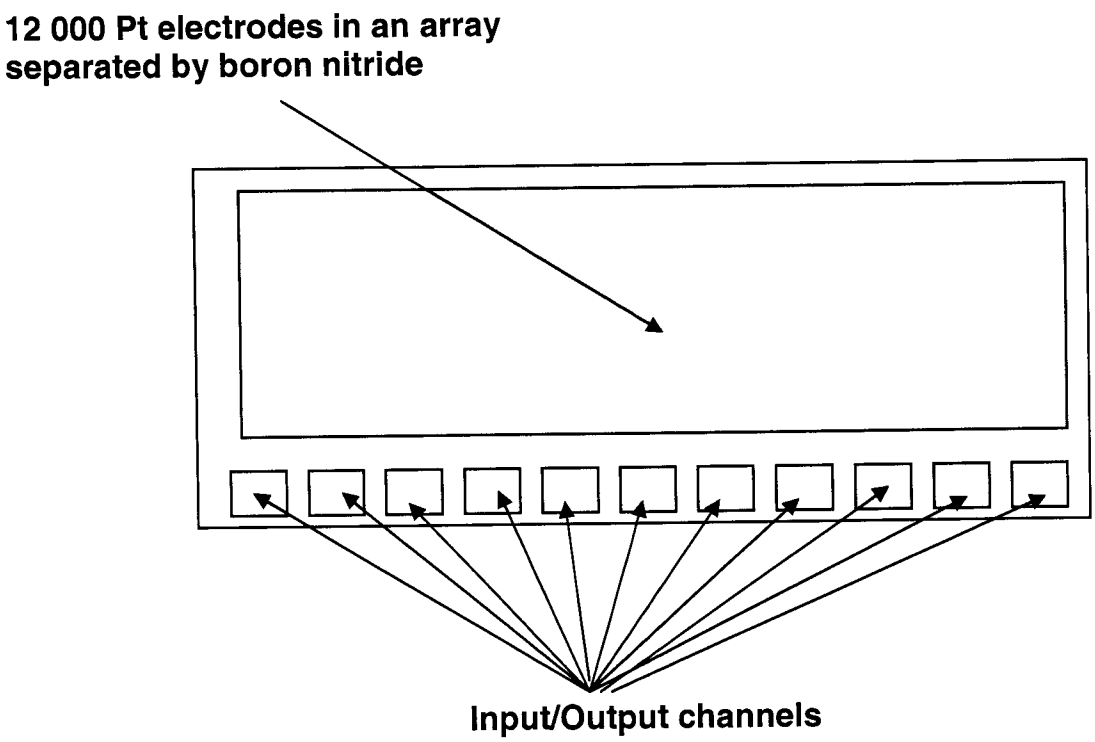


Figure 6.6 Schema of the CombiMatrix CMOS microarray.

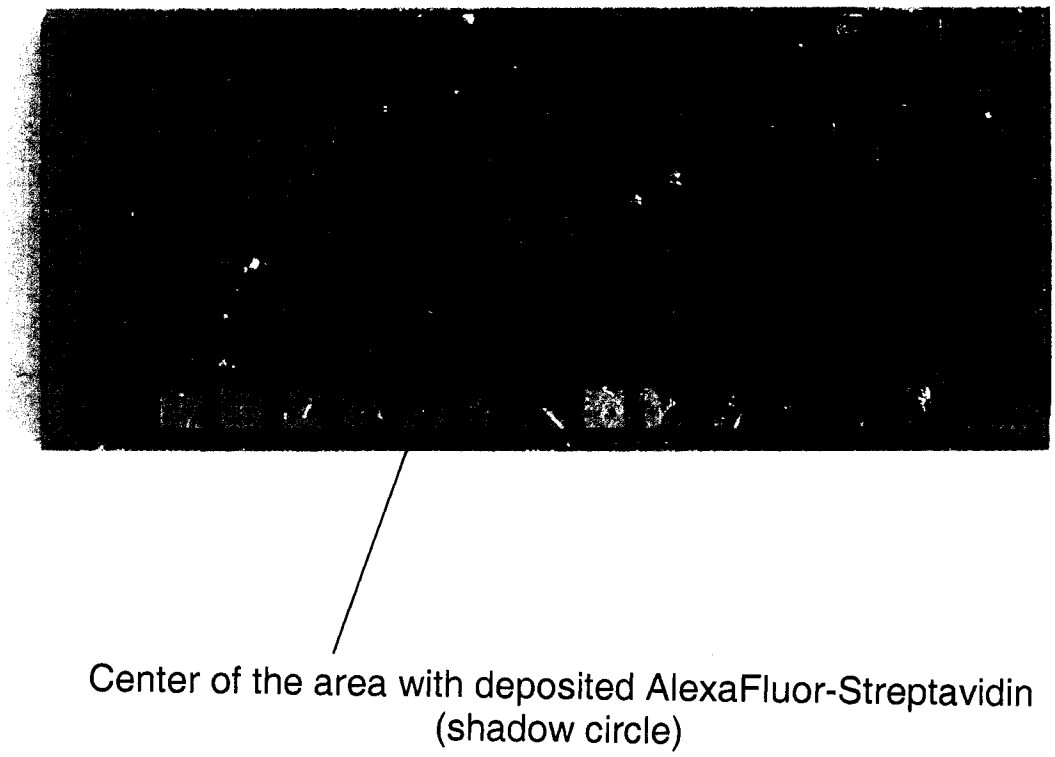


Figure 6.7 Picture of the CombiMatrix CMOS microarray with landed protein.

Spot of landed fluorescence labeled streptavidin is visible on the array.

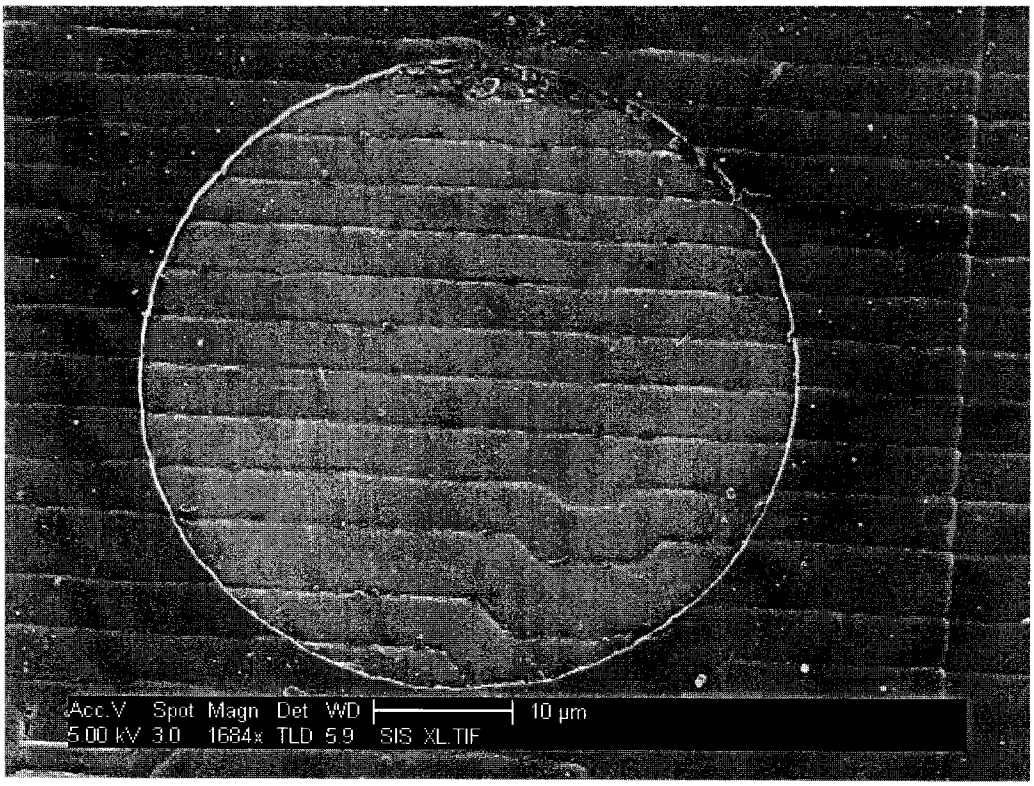


Figure 6.8 SEM of a single electrode in the array on the Combimatrix chip.

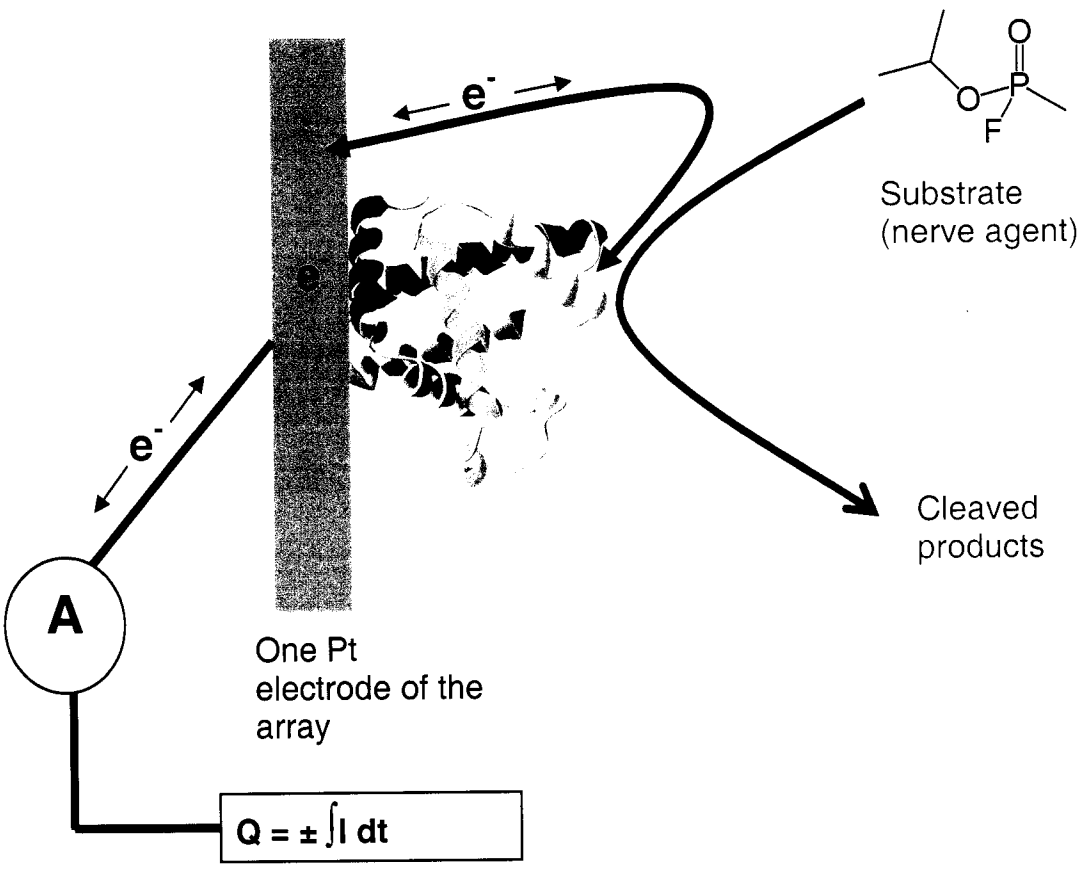


Figure 6.9 Nerve agent detection scheme.

Schematic diagram of detection scheme that utilizes immobilized organophosphorus acid anhydrolase.

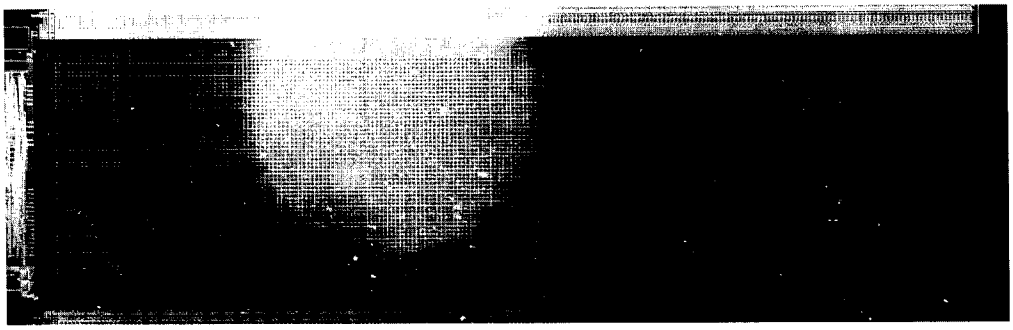
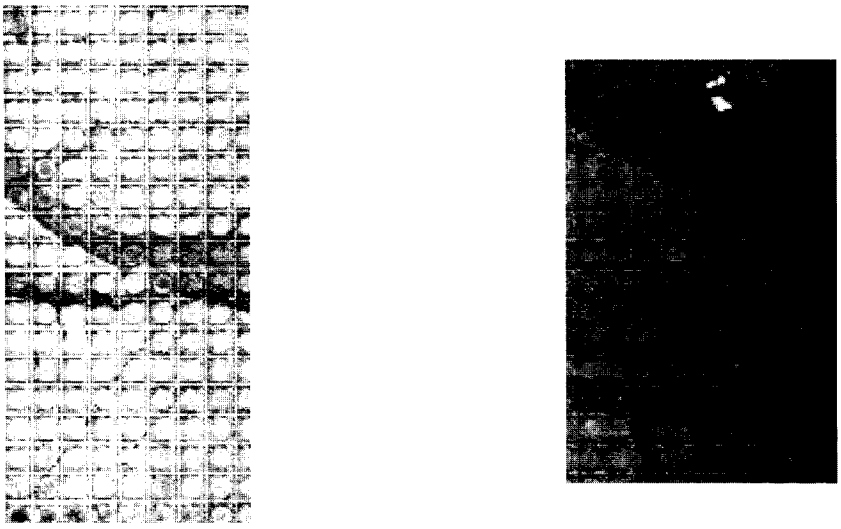


Figure 6.10 Fluorescence scan of modified array.

Fluorescence scan of chip with landed streptavidin fluorescence conjugate after washing with deionized water



CY-5 Labeled biotin attached to Streptavidin

A

Out of streptavidin deposition area

B

Figure 6.11 Fluorescence scan of modified array.

Fluorescence from the chip exposed to the solution of biotin-cy5 conjugate A) area with previously deposited streptavidin B) blank area.

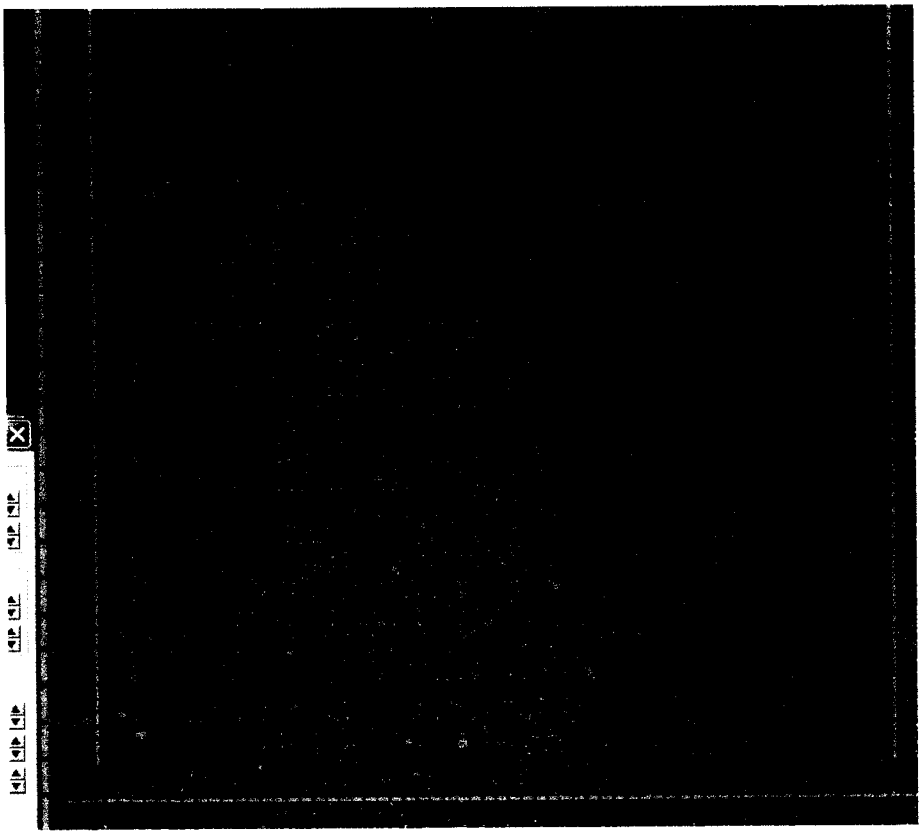


Figure 6.12 Fluorescence from individual electrodes of the array.

Fluorescence from electrodes modified by reactive landing. The chip on the figure is fully functional and all the electrodes successfully passed addressing tests.

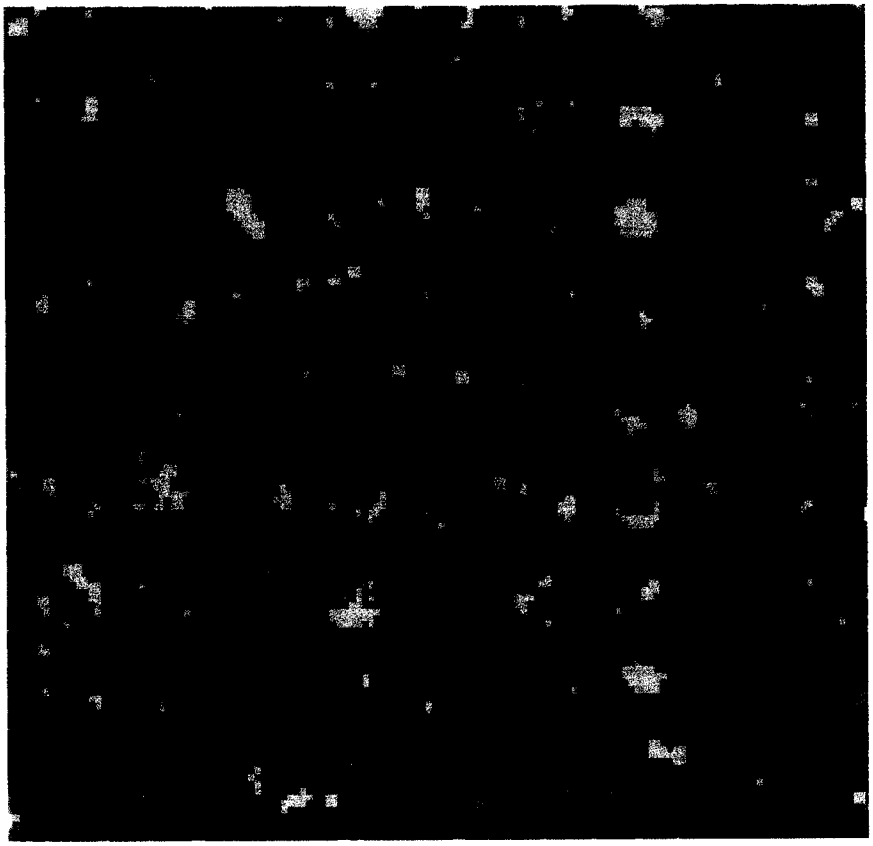


Figure 6.13 Detailed scan of Figure 6.12.

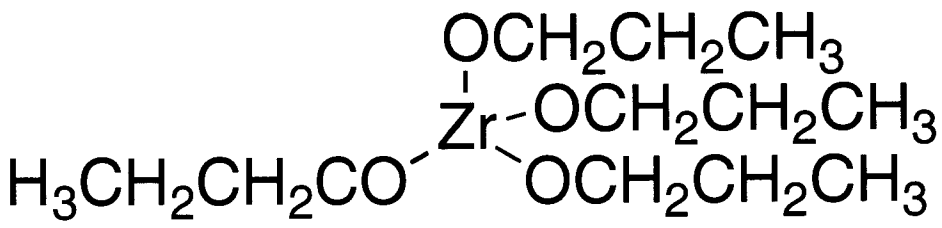


Figure 6.14 Zirconium propoxide.

Table 6.1 XPS Analysis of metal surfaces with landed hyaluronan.

Low and high resolution XPS spectra of single spot area[25] and large area covered with hyaluronan. Composition is in atomic %.

	Single spot[a]	Large area
O	31.8	32.6
N	3.7	2.8
C	55.2	57.9
Carbon 1s 285.0 eV	37.0	36.8
Carbon 1s 286.7 eV	39.5	42.4
Carbon 1s 288.3 eV	19.0	18.0
Carbon 1s 289.8 eV	4.5	2.7

[a] reported in ref. 25, Tab II, column A

Table 6.2 XPS Analysis of metal surfaces with landed hyaluronan after washing.

Different washing procedures test robustness of hyaluronan coating. XPS of blank stainless steel surface is listed for comparison. Composition in atomic %. (nd = not detected)

	Coated samples				Blank
Washing solution	Water	NaCl	Urea	Water	Water
Washing time (min)	5	30	30	30	30
C	63.9	54.1	66.9	62.8	33
O	27.7	38.8	26.8	29.8	52.4
N	2.7	3.1	1.3	4	nd
Fe	3.8	2.1	2.3	0.4	12
Cr	0.2	nd	0.7	nd	2.5

Table 6.3 XPS of landed zirconium propoxide.

XPS Composition scan of landed zirconium propoxide. All samples were washed before XPS analyses. Composition in atomic % (nd=not detected)

Sample description	C	O	Fe	Cr	Zr
Sample 1 Inside landed spot	40.5	45.8	nd	nd	13.7
Sample 1 [a]Blank area	40.3	46.9	9.6	1.7	nd
Sample 2 Inside landed spot	47.5	40.2	nd	nd	10.6
Sample 2 [b]Blank area	50.8	39.5	7.8	0.6	nd

[a]Intact area
[b]Area where zirconium propoxide was dried from solution

6.5 Notes to Chapter 6

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APPENDIX: PEER-REVIEWED PUBLICATIONS FOCUSED ON SOFT AND REACTIVE LANDING FROM TUREČEK LABORATORY

Publications based on the presented dissertation:

P1. Volný, M.; Elam, W. T.; Branca, A.; Ratner, B. D.; Tureček, F., **Preparative soft and reactive landing of multiply charged protein ions on a plasma-treated metal surface.** *Anal. Chem.* **2005**, *77*, 4890-4896.

P2. Volný, M.; Elam, W. T.; Ratner, B. D.; Tureček, F. **Preparative Soft and Reactive Landing of Gas-Phase Ions on Plasma-Treated Metal Surfaces.** *Anal. Chem.* **2005**, *77*, 4846-4853.

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VITA

Michael Volný was born in Prague, Czechoslovakia (now Czech Republic) to a librarian and science-fiction novelist. He entered the public school system at Základní Škola Křejpského in Prague Jižní Město district and continued his education at Gymnázium Na Vítězné pláni. After graduating from high school, he started to study science at the Charles University in Prague and informatics and statistics at the Prague School of Economics. His first interest was business software engineering and in 1995 he published a bestseller about the effective use of Czech programs and software tools. Nevertheless, he eventually decided to major in chemistry and graduated from the Charles University in early 1997. He began work as an assistant of Dr. Petr Pithart, the conservative Speaker of the Senate of the Czech Parliament. Though several career possibilities in governmental sector were offered to him, the close experience with the real political process actually made Michael to return from renaissance and baroque palaces to the laboratory. He started working on a research project and in 1999 he obtained Master of Science under Dr. Petr Rychlovský in the field of atomic spectroscopy. Michael hoped to proceed with his graduate education at the Charles University towards his doctorate but that plan eventually failed. Thus, he had to try his luck at different West European and North American universities. He succeeded and quickly received another Master of Science in chemistry this time at the University of Washington in Seattle with overall GPA 3.80. In 2006, he earned a Doctor of Philosophy degree under the supervision of Dr. František Tureček at the same university in the fields of surface science and mass spectrometry and won the 2006 *Journal of Mass Spectrometry* award for his work on spectroscopy of polyatomic ions. While in graduate school in Seattle, Michael married Dr. Petra Volná, an immunologist at the University of Washington Department of Pediatrics and Seattle Childrens Hospital.