



The adsorption of the mussel adhesive proteins of the marine mussel, *Mytilus edulis*, to polymer films
by Ace M Baty

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Chemical Engineering
Montana State University
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Abstract:

The adsorption of mussel adhesive protein (MAP) from the marine mussel *Mytilus edulis* has been investigated on polystyrene (PS) and poly(octadecyl methacrylate) surfaces using angle dependent x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). Images acquired in the dehydrated state using contact mode AFM are compared with images acquired in the hydrated state using fluid Tapping Mode™ to assess the contribution that dehydration has on the adsorbed proteins. To further assess the stability of the adsorbed protein layer, XPS analysis was performed at liquid nitrogen (LN2) temperature without dehydrating the samples and at room temperature after the surfaces were dehydrated. The adsorption pattern of MAP is distinctly different on the two polymer surfaces when imaged in the hydrated state. The adsorption pattern of MAP in the dehydrated state, revealed by AFM images, is similar to the hydrated images but shows a loss of structure and spatial distribution of the adsorbed proteins. MAP adsorbed to PS showed a collapse of the adsorbed proteins, towards the surface, upon dehydration, but no loss in lateral spatial distribution. In contrast, MAP adsorbed to POMA showed a loss of lateral spatial distribution upon dehydration. Angle resolved XPS shows differences in nitrogen composition with depth for MAP adsorbed to PS and POMA at liquid nitrogen temperature. Angle resolved XPS at room temperature shows significant differences over the LN2 temperature studies indicating that hydration plays an important role in stabilizing the adsorbed protein at the surface. The differences observed upon dehydration can be attributed to the strength of the interactions between the adsorbed MAP and the surface. The AFM and XPS data indicate that the adsorbed MAP is stabilized on the surface of the PS through specific interactions preventing the protein from losing little of its lateral spatial distribution across the surface. The adsorbed MAP on the POMA indicates a loosely bound protein layer that is adsorbed through non-specific types of interactions allowing the protein to lose much of its lateral spatial distribution when dehydrated. This data demonstrates that the chemistry of the polymer film that is present at the protein-polymer interface can influence protein-protein and protein-surface interactions.

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Ace M. Baty III

A thesis submitted in partial fulfillment of the requirements
for the degree

of
Master of Science
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MONTANA STATE UNIVERSITY
Bozeman, Montana

November 1995

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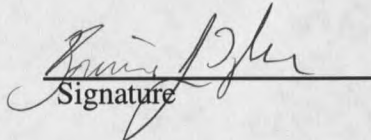
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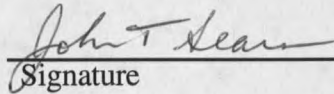
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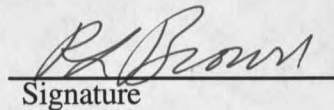
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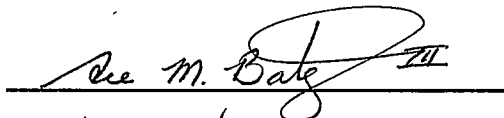
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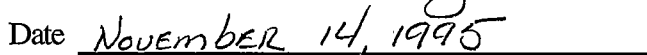
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This work is dedicated to Kristi and Ellie

ACKNOWLEDGMENTS

I would like to express my greatest appreciation and gratitude towards my committee members, Dr. Gill Geesey, Dr. Bonnie Tyler, Dr. Peter Suci and Dr. James Bryers. They have entertained my thoughts, ideas and interpretations on many occasions with enduring patience.

I would like to thank Dr. Joe Gardella Jr. at SUNY Buffalo, Dr. Pat Schamberger at Montana State University and Deborah Leach-Scampavia at NESA/BIO at the University of Washington for their guidance and expertise in x-ray photoelectron spectroscopy (XPS). I would also like to express my gratitude to Dr. Pamela Leavitt and Dr. Larry Davis at Physical Electronics Inc. in Minneapolis, MN for their help with the cold probe XPS experiments. Without their help and generous gift of many hours of instrument time this thesis would not have been completed. Thanks are extended to Kevin Siedlecki at MSU and Barney Drake and Clint Calahan at Imaging Services Inc. in Santa Barbara, CA for their help in atomic force microscopy (AFM) imaging. I would also like to thank Dr. Roger Marchant and Chris Siedlecki at Case Western Reserve University for the use of their laboratory and knowledge in hydrated AFM imaging.

My research has been sponsored by the Office of Naval Research under Grant N0014-93-1-0168, and the NSF under cooperative agreement EEC 8907039 and a grant to Dr. Gill G. Geesey from 3M.

Finally, I would like to acknowledge with love and gratitude the support of my wife, Kristi and my daughter Ellie. Also my parents, Ace and Ann and Kristi's Mom, Carol, my Grandmother Robinson and my beloved late Aunt Mary, who, through their love and support enabled me to accomplish my goals.

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Abstract

The adsorption of mussel adhesive protein (MAP) from the marine mussel *Mytilus edulis* has been investigated on polystyrene (PS) and poly(octadecyl methacrylate) surfaces using angle dependent x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). Images acquired in the dehydrated state using contact mode AFM are compared with images acquired in the hydrated state using fluid Tapping ModeTM to assess the contribution that dehydration has on the adsorbed proteins. To further assess the stability of the adsorbed protein layer, XPS analysis was performed at liquid nitrogen (LN₂) temperature without dehydrating the samples and at room temperature after the surfaces were dehydrated. The adsorption pattern of MAP is distinctly different on the two polymer surfaces when imaged in the hydrated state. The adsorption pattern of MAP in the dehydrated state, revealed by AFM images, is similar to the hydrated images but shows a loss of structure and spatial distribution of the adsorbed proteins. MAP adsorbed to PS showed a collapse of the adsorbed proteins, towards the surface, upon dehydration, but no loss in lateral spatial distribution. In contrast, MAP adsorbed to POMA showed a loss of lateral spatial distribution upon dehydration. Angle resolved XPS shows differences in nitrogen composition with depth for MAP adsorbed to PS and POMA at liquid nitrogen temperature. Angle resolved XPS at room temperature shows significant differences over the LN₂ temperature studies indicating that hydration plays an important role in stabilizing the adsorbed protein at the surface. The differences observed upon dehydration can be attributed to the strength of the interactions between the adsorbed MAP and the surface. The AFM and XPS data indicate that the adsorbed MAP is stabilized on the surface of the PS through specific interactions preventing the protein from losing little of its lateral spatial distribution across the surface. The adsorbed MAP on the POMA indicates a loosely bound protein layer that is adsorbed through non-specific types of interactions allowing the protein to lose much of its lateral spatial distribution when dehydrated. This data demonstrates that the chemistry of the polymer film that is present at the protein-polymer interface can influence protein-protein and protein-surface interactions.

Chapter 1

Introduction

1.1 Background

It has been shown that conditioning films form on virtually every surface when they are immersed in a natural aqueous environment, be it the human body, fresh bodies of water, estuarine waters or the open ocean (Little, 1985; Marzalek, Gerchakov and Udey, 1979; Corpe, 1977; Niehof and Loeb, 1973). This conditioning film is comprised of mostly proteinaceous materials (Baier, 1980). Previous studies have indicated that conditioning films can mask the underlying substratum chemistry (Marshall, 1992) but other studies have shown that it can also reflect differences in surface chemistry of the underlying substratum (Healy, Thomas, Rezaei, Kim, McKeown, Lom and Hockberger, 1995). The conditioning film is a major uncertainty in the area of biofouling. Any surface designed specifically for its nonfouling properties will be covered with a conditioning film. Microorganisms attach to the conditioning film via proteinaceous and exopolysaccharide structures, often bypassing an engineered "minimally adhesive" surface. One major problem in studying the microbial adhesion to the conditioning film is that this film is a completely undefined surface for the study of initial adhesion events. The mussel adhesive proteins (MAP), MeFP-1 and MeFP-2, are attractive model conditioning films for the study of these events because of their well characterized chemistry. This research explores the adsorption of these proteins and attempts to characterize the interactions responsible for MAP adhesion to low energy polymer surfaces that display different surface chemistries. Once these types of protein-surface interactions are characterized, the information can be used to understand interactions with holdfast structures of fouling microorganisms and

perhaps offer the opportunity to design surfaces that resist protein adhesion and subsequent biofouling.

The marine mussel, *Mytilus edulis*, produces a series of adhesive proteins that allow the organism to attach itself to a variety of surfaces in an underwater environment (Waite, 1987). These proteins serve to support and bind components of the adhesive holdfast composed of byssal threads (Waite, 1983). The byssal threads are comprised of a collagenous matrix that is secreted by the collagen gland (Vitellaro-Zuccarello, 1980). Four proteins have been identified in the byssal threads that are thought to serve these structural and adhesive functions; *Mytilus edulis* Foot Proteins (MeFP), 1, 2, 3 and 4, collectively termed Mussel adhesive proteins (MAP).

The MeFP-1 has been determined to be a 130 kD protein consisting of tandemly repeated decapeptide sequences, shown in Figure 1 (Waite, Housley and Tanzer, 1985).

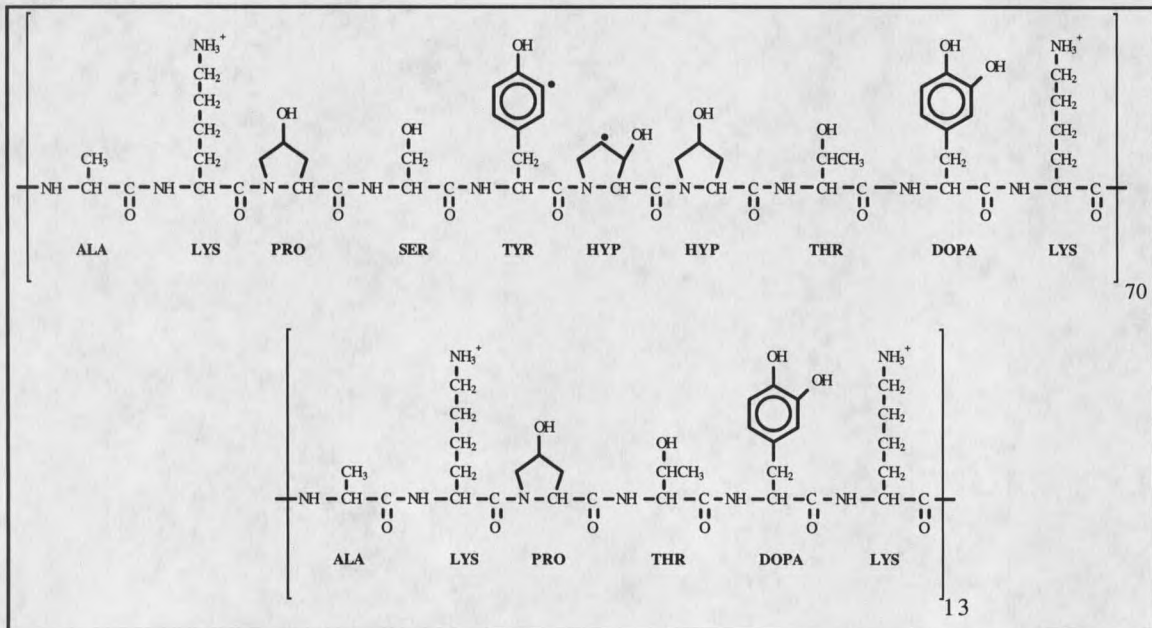


Figure 1. Tandemly repeated deca- and hexa-peptide sequences of MeFP-1, Dots indicate points of optional hydroxylation.

MeFP-1 has extensive hydroxylation of tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and of proline to hydroxyproline (HYP) (Waite and Tanzer, 1980; Waite, Housley and Tanzer, 1985). This protein is unusual as it is one of the first proteins discovered to contain DOPA in its primary sequence and is one of the few proteins found in nature that contain hydroxyprolines in non-collagenous sequences (Taylor, Ross, Shabanowitz, Hunt, Waite, 1994). The hydroxyprolines impart a slight circular dichroism, present as 20% β -turns, due to the hydroxyprolines but imparts no other secondary structure (Williams, Marumo, Waite and Henkens, 1989). MeFP-1 is secreted by the phenol glands in the muscular foot and is cross-linked by an enzyme during sclerotization (Waite, 1990; Rzepecki and Waite, 1991). MeFP-1 is present on the surface of the byssal threads as a protective varnish and is thought to play an integral role in the actual attachment of the byssal thread to the surface (Waite, 1983) (Benedict and Waite, 1986).

The MeFP-2 is a 42-47 kDa protein that is tandemly repetitive with at least three motifs (Rzepecki, Hansen and Waite 1992). The structure of MeFP-2 is much more difficult to represent since its sequence degeneracy is much greater than in MeFP-1. In contrast to MeFP-1, MeFP-2 contains 6-7 mol% of the disulfide containing amino acid cystine, indicating considerable secondary structure. It has been suggested that MeFP-2 serves a structural function, comprising 25% of the plaque protein of the byssal thread.

The MeFP-3 is a 6-7 kDa protein that has 20 mol% of DOPA. Its structure has a very high degree of degeneracy and has been speculated that the mussel can control this at a surface specific level (Papov, Diamond, Biemann and Waite, 1995). MeFP-3 has a high degree of degeneracy, a low molecular weight and a high mol % of DOPA which might suggest its function as a surface primer. However its exact function has yet to be determined. Likewise the structure and function of the MeFP-4 protein has not yet been fully characterized.

There are four mechanisms, shown in figure 2, that have been proposed to play important roles in MAP-MAP and MAP-surface interactions: hydrogen bonding (a), metal-ligand complexes (b), Michael-type addition compounds derived from o-quinones (c), and charge transfer complexes (d) (Waite, 1987).

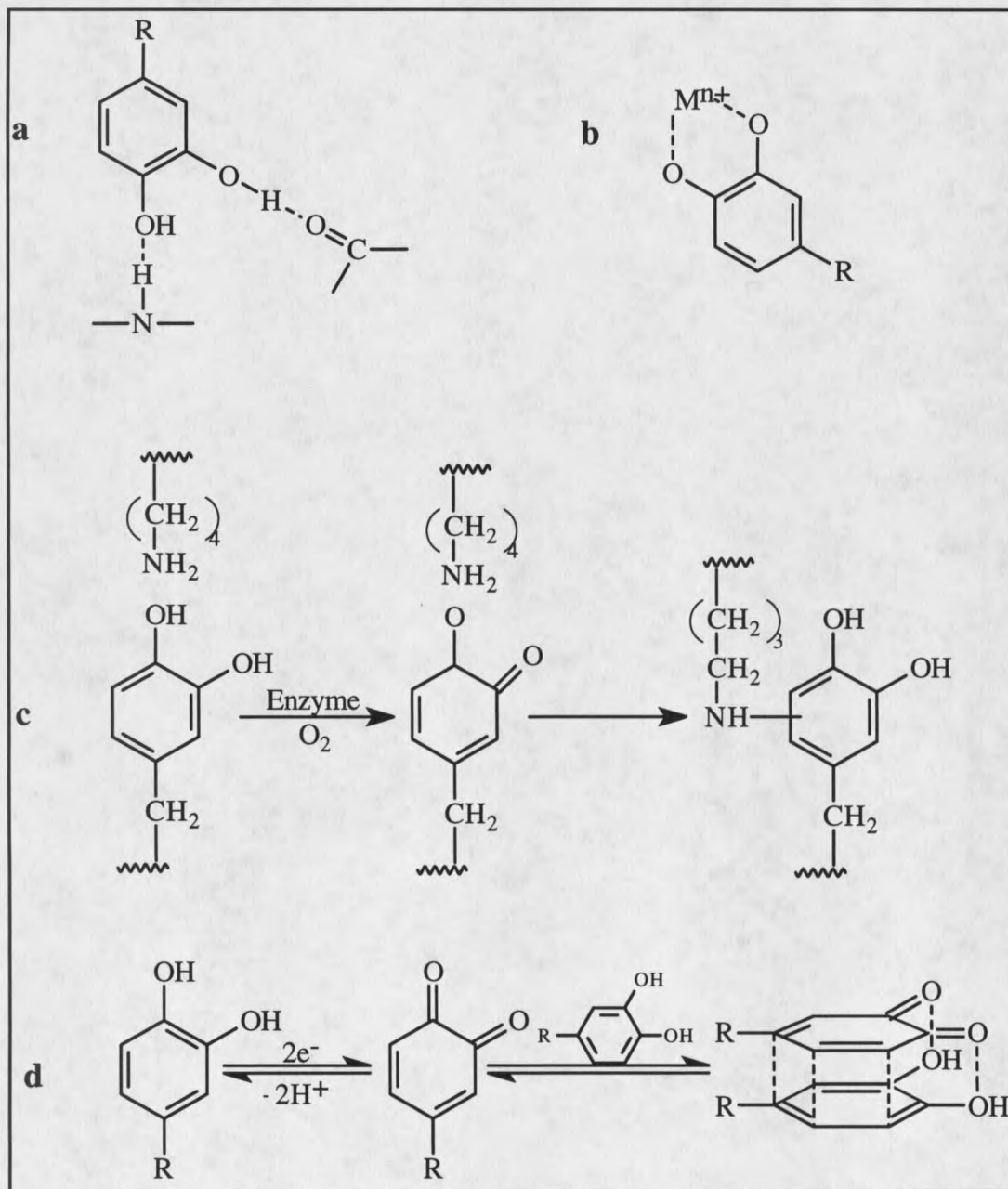


Figure 2. Interactions involving the DOPA residue of MeFP-1.

Olivieri (Olivieri, Loomis and Baier 1992) has collected data that suggests MAP can orient itself towards oxide surfaces enabling the L-DOPA residues to interact with the surface through hydrogen bonding. Hansen (Hansen, Luther and Waite, 1994) has recently found that MAP interacts with stainless steel by complexing and binding with surface metals. The Michael-type addition compounds are driven by the catechol oxidase enzyme that is co-secreted with the proteins in the natural system (Waite, 1989). The existence of the charge transfer complex has never been shown directly for this system, but has been shown to occur in proteins with similar quinone-chemistry.

Commercial uses for MAP have been limited, due to the lack of understanding of how these proteins function as an adhesive. However, these proteins have seen recent interest in the biomedical community as a tissue adhesive. MAP has been studied for its ability to fix chondrocyte allografts internally (Pitman, Menche, Song, Ben-Yishay, Gilbert and Grande, 1989; Grande and Pitman, 1988). The protein has also been used in experimental epikeroplasty in laboratory animals (Robin, Picciano, Kusleika Salazar and Benedict, 1988), as well as an adhesive agent to increase cellular attachment to substrata (Olivieri, Rittle, Tweden, and Loomis, 1992). Recent studies have also indicated that MAP enhances the attachment of osteoblasts and epiphyseal cartilage cells to substrata (Fulkerson, Norton, Gronowicz, Picciano and Massicotte, 1990). However, these studies have only been met with marginal success, as the actual anchoring mechanism of MAP is poorly understood and despite MAP's tenacious adhesive action in the natural environment, this function has yet to be duplicated with the purified proteins out of their natural environment. A greater understanding of how MAP binds to surfaces is essential in developing MAP as a useful tissue adhesive.

1.2 Detection of MAP Adsorption Phenomena

Since protein adhesion to surfaces occurs in an aqueous environment, any reliable chemical analysis of the structure of the adsorbed proteins must be performed in the hydrated state. It is widely accepted that desiccation can drastically affect the structure of biological macromolecules (Lehninger, Nelson and Cox, 1993). Unfortunately, the study of hydrated surfaces is not compatible with many ultra-high vacuum (UHV) surface analysis techniques such as x-ray photoelectron spectroscopy (XPS). Therefore cryostage sample handling techniques must be employed during the analysis of a hydrated surface in UHV. A hydrated surface can be frozen at liquid nitrogen (LN_2) temperatures and loaded onto a cold stage where the sample can be kept at LN_2 temperatures during analysis (Ratner, Weathersby, Hoffman, Kelly and Sharpen, 1978; Ratner and Castner, 1994). The structure of the adsorbed molecules at the surface are preserved in their hydrated state. Some sublimation of adsorbed water does occur but the sample is not pumped completely dry, thereby locking the structure of the protein in its hydrated state.

Different problems are encountered when attempting to image adsorbed proteins in fluid using atomic force microscopy (AFM). Here the problem is not with the sample preparation but the method employed to do the imaging. Adsorbed biological molecules are extremely fragile when exposed to the large forces exerted by the AFM probe tip during fluid contact mode imaging. To remedy this problem a mode of operation called Tapping ModeTM is used. In Tapping ModeTM the AFM cantilever operates at a resonant frequency that is characteristic of the attractive-repulsive forces that are acting between the AFM probe tip and the surface (Zhong, Inniss, Kjoller and Elings, 1993). Tapping ModeTM is characterized by overall weak tip-sample interactions. This allows minimal disturbance of the adsorbed molecules being imaged. Studying protein adsorption using AFM imaging has one special attribute; it can obtain direct information about protein adsorption with a

high lateral and high vertical spatial resolution. Thus, questions of the spatial distribution of proteins at submonolayer to monolayer surface coverages can be answered, thereby allowing direct information of the initial spatial distribution and density of the nucleation events on the surface to be determined. With the advent of techniques such as fluid Tapping ModeTM using AFM and cryostage techniques in XPS new insights can be anticipated from the analysis of hydrated, adsorbed proteins.

1.3 Goals and Objectives

The goal of this study is to investigate the adsorption of mussel adhesive protein (MAP) to polystyrene (PS) and poly(octadecyl methacrylate) (POMA) surfaces in the dehydrated state using variable angle XPS and AFM imaging and in the hydrated state using cold probe techniques in variable angle XPS and AFM imaging using fluid Tapping ModeTM. XPS will quantify the elemental composition with depth of the adsorbed protein and AFM will provide information about the architecture of the protein film adsorbed to the polymer surfaces.

Chapter 2

Analytical Methods

2.1 X-ray Photoelectron Spectroscopy

2.1.1 Photoelectron Emission

X-ray photoelectron spectroscopy is an analytical surface analysis technique that irradiates a sample with an x-ray source and induces the ejection of photoelectrons from the surface of the sample (Carlson, 1975; Briggs, 1977; Fadley, 1981). The x-ray source is commonly a Mg or Al anode that is bombarded with high energy electrons (≈ 10 keV) from a heated filament or by an electron gun. This produces x-ray emissions that are characteristic of the anode; either Mg $K\alpha$ or Al $K\alpha$ x-rays. The x-rays bombard the surface being studied and produce the emission of photoelectrons by the photoelectron process (Atkins, 1990). During the photoelectron process, an atom is irradiated by the x-ray source which causes the photoionization of core electrons, as shown in Figure 3. The resulting photoelectrons have a kinetic energy that is equal to the x-ray energy minus the binding energy of the photoelectrons minus the work function of the spectrometer, shown in Equation 1 (Christie, 1990)

$$K.E. = h\nu - B.E. - \phi_w \quad (1)$$

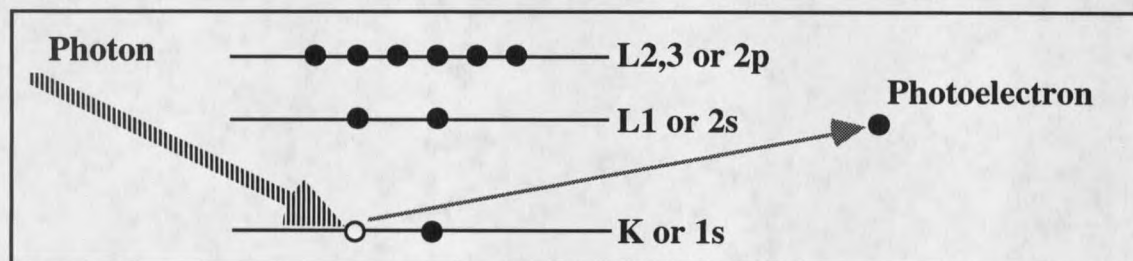


Figure 3: Diagram of the photoelectric process

The photoelectron that emerges from the surface has a binding energy that is characteristic of the environment from which it came. For example, the resulting photoelectron from a carbon atom singly bound to an oxygen atom will emerge at a slightly different binding energy than a carbon atom doubly bound to an oxygen atom. A list of the binding energy (B.E.) positions, that are used in this research, for a variety of oxidation states of carbon and oxygen are given in Table 1 (Ratner and Castner, 1994).

Table 1: Binding Energy Positions for Carbon and Oxygen

Name	Functionality	B.E. (eV)
hydrocarbon	C—H C—C	285.0
amine	C—N	286.0
alcohol ether	C—OH C—O—C	286.5
Cl bound to Carbon	C—Cl	286.5
carbonyl	C=O	288.0
amide	N—C=O	288.2
acid ester	O—C=O	289.0
urea	$\begin{array}{c} \text{O} \\ \\ \text{N}-\text{C}-\text{N} \end{array}$	289.2
carbamate	$\begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{N} \end{array}$	289.9
carbanate	$\begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{O} \end{array}$	290.3
carbonyl	C=O O—C=O	532.2
alcohol ether	C—OH C—O—C	532.8
ester	C—O—C=O	533.7

Most ranges are $\pm 2\text{eV}$

For photoionization to take place, the energies of the electrons emitted cannot exceed the energy of the ionizing photons. This means the B.E. of the photoelectron cannot exceed the energy of the X-ray ($h\nu$) for photoionization to occur. The probabilities of interaction of the emitted electrons with the surface material far exceed those of the photons from the X-ray source. Therefore, while the path length for the photons are several micrometers, the path length for the emitted electrons are within tens of angstroms. So while ionization occurs to a depth of a few micrometers only the electrons emitted within a few tens of angstroms of the surface can leave the surface and be detected without energy loss. This is termed an inelastic scattering event.

2.1.2 Inelastic Mean Free Path and Sampling Depth

An inelastic scattering event depends on the energy of the photoelectron and the material through which that photoelectron must travel. Since this is a process governed by probability, an inelastic scattering event can be described by the standard exponential decay law, shown in Equation 2 (Christie, 1990).

$$I(x) = I_o \exp \left[\frac{-x}{\lambda_{(E_{kz})} \cdot \cos(\theta)} \right] \quad (2)$$

Where $I(x)$ represents the signal intensity of the photoelectrons after traveling through a material of thickness, x , at an angle θ , with respect to the surface normal. The variable λ_n represents the inelastic mean free path (IMFP) of the photoelectron through the material. Due to the exponential behavior predicted by Equation 2, it is very difficult to arrive at a unique sampling depth. However, 63% of the photoelectrons must originate from a distance that is the product of the IMFP and the cosine of the take-off angle, with respect to the surface normal, of the photoelectrons, as shown in Equation 3 (Christie, 1990).

$$d = \lambda_n \cos(\theta) \quad (3)$$

Likewise, 86% of the photoelectrons originate from a distance of $2d$ within the sample and $3d$ represents 95% of the signal intensity. For most practical purposes, $3d$ is used as the depth sampled in XPS (Christie, 1990).

2.1.3 Variable Angle XPS

The relationship between the depth sampled and the take-off angle of the photoelectrons can be represented schematically in Figure 4.

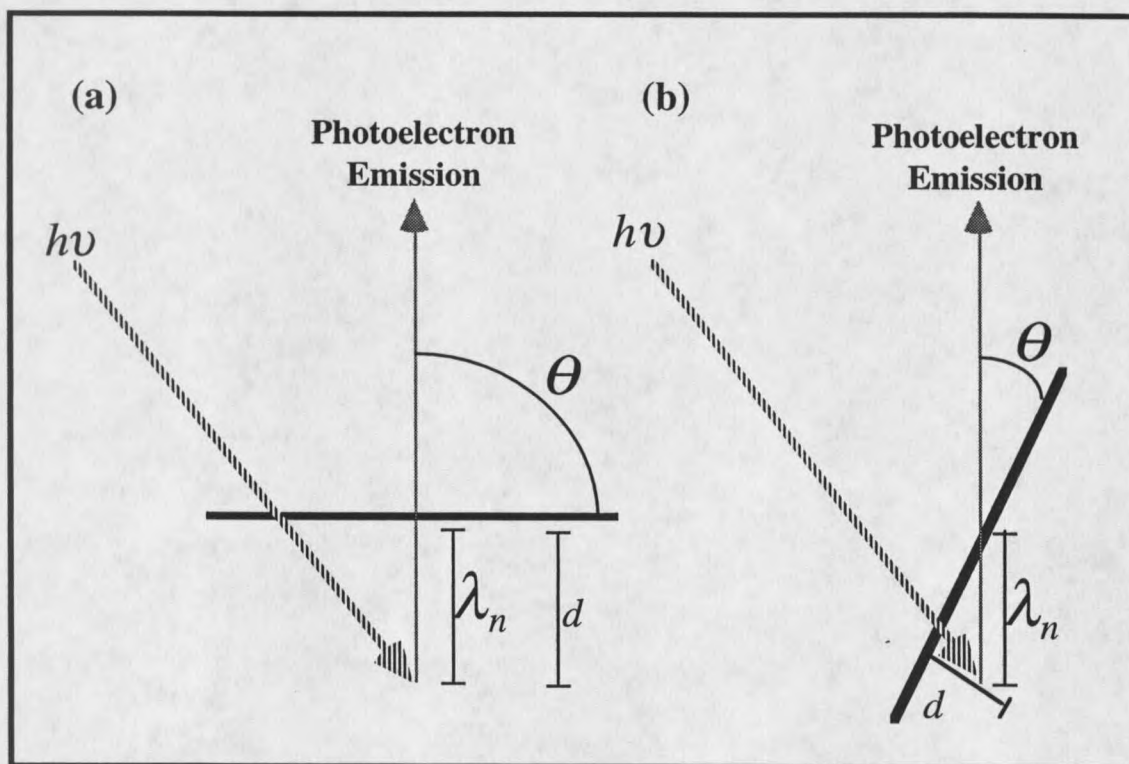


Figure 4: Schematic representing the relationship between high and low take-off angles (θ), inelastic mean free path (λ_n) and sampling depth (d).

As the angle from the surfaces decreases the photoelectrons that originate from the upper few angstroms of the sample must travel a greater distance through the sample to reach the detector. Therefore, as the take-off angle decreases the sampling depth decreases. To estimate this sampling depth, the only unknown parameter in equation 3 is the IMFP or λ_n . The IMFP has been previously described by Equation 4, which accurately describes the IMFP for organics, inorganics and elemental species (Seah and Dench, 1980)

$$\lambda_n = \frac{A_n}{(K.E.)^2} + B_n (K.E.)^{\frac{1}{2}} \quad (4)$$

The IMFP, λ_n , is calculated in nanometers. A_n and B_n are curve fit parameters that have been previously determined for organics, inorganics and elemental species and can be obtained in the literature (Seah and Dench, 1980). The variable, K.E., is the kinetic energy of the photoelectron being detected and can be calculated from Equation 1. Values for the escape depths of carbon, oxygen and nitrogen, using curve fit parameters from organic compounds and an x-ray energy of 1486.5 eV (aluminum $K\alpha$ x-ray source) are shown in Table 2.

Table 2: Calculated Escape Depths (3d)

Angle (surface normal)	C ($\text{\AA}$)	N ($\text{\AA}$)	O ($\text{\AA}$)	Angle (surface)
10	88.96	84.64	79.27	80
55	51.81	49.30	46.17	35
68	33.84	32.20	30.15	22
75	23.38	22.25	20.83	15
80	15.69	14.93	13.98	10

2.2 Atomic Force Microscopy

2.2.1 AFM Detection Technique

Atomic Force Microscopy (AFM) is a surface analysis technique that is capable of imaging the topography and frictional forces of a samples surface (Binnig, Quate and Gerber, 1986). The AFM operates by rastering a probe tip over the surface being studied. As the probe tip approaches the surface, forces are exerted on the probe tip. This causes a small deflection in the cantilever that is attached to the probe tip. As the probe tip is rastered across the surface the deflection of the cantilever remains proportional to the surface features on the samples surface. This deflection is most easily and most commonly measured by a laser photodiode detector, as is shown in Figure 5 (Prater and Strauser, 1994).

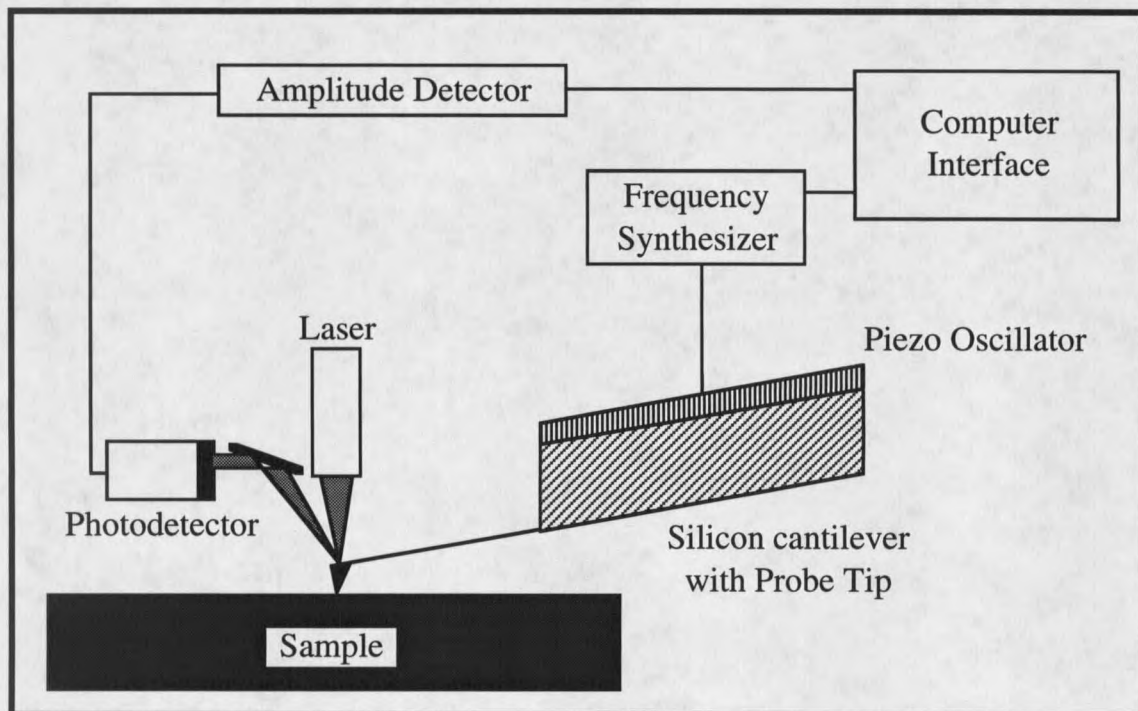


Figure 5: Schematic of AFM components.

This detection technique employs a laser beam that is deflected off the tip of the cantilever. As the cantilever is deflected the position of the laser striking the photodiode changes. This is translated via a computer interface to length, width and height information. In conjunction with topographical information the AFM also calculates the force exerted on the probe tip by using the spring constant of the cantilever.

2.2.2 *Contact Mode AFM*

There are three operational modes of the AFM (Prater and Strauser, 1994); Contact Mode, Non contact Mode and Tapping Mode. Contact mode is the simplest mode of operation (Sarid, 1991). During this mode the AFM probe tip is actually brought into contact with the surface and the cantilever is deflected by defects in the surface. Topography is directly measured from the surface to the probe tip. The most common problem with this mode of operation is excessive forces applied by the probe tip (Drake, Prater, Weisenhorn, Gould, Albrecht, Quate, Cannell, Hansma and Hansma, 1989). Biological samples are typically very labile and delicate and excessive force applied by the probe tip can damage the surface being studied and introduce artifacts into the image. The effects of a very large applied force can be minimized by reducing the applied force but there are limits to this in an ambient environment due to adsorbed water on the surface. When a sample is imaged under ambient conditions, a monolayer of water and gases adsorb to the surface. This monolayer can form a meniscus with the cantilever probe tip, pulling it into the surface with a force of approximately 100 nN. This force puts a limit on the reduction of the applied force. An additional attractive force can be contributed by electrostatic charge that builds up on insulating and semiconducting samples as the probe tip is rastered across the surface. All of these surface-tip forces can supply a substantial frictional force to the sample surface, which in the case of very delicate surfaces, can

damage or even destroy the surface. However, for many applications, contact mode AFM is the fastest and easiest mode of operation.

2.2.3 *Non-contact Mode AFM*

The second mode of operation is the non-contact mode (Martin, Williams and Wickramasinghe 1989). During this mode of operation the probe tip is oscillated above the surface of the sample. The probe tip then measures the force gradients across the surface as a change in amplitude, phase or frequency of the oscillation. The forces that can be measured by this technique include Van der Waals forces, magnetic forces and electrostatic forces. Attempts to use non-contact mode AFM are generally very difficult, as the AFM probe tip can be trapped in the fluid monolayer at the surface of the sample. This makes it very difficult to approach the surface to measure the necessary forces.

2.2.4 *Tapping ModeTM AFM*

Tapping ModeTM AFM is a mode of operation that can achieve a high resolution without damaging the surface with excessively high frictional forces (Zhong, Inniss, Kjoller and Elings, 1993). In tapping mode, the AFM cantilever and probe tip are oscillated at their resonant frequency over the surface of the sample. As the probe tip is brought into close proximity to the surface the tip begins to tap the surface. As the tip periodically contacts the surface the oscillation amplitude is dampened. A feedback controller continually adjusts the frequency to maintain a constant amplitude as the tip is scanned across the surface. Since the probe tip only intermittently taps the surface, the frictional forces exerted on the surface are negligible. The probe tip is prevented from being trapped in the fluid monolayer by setting the oscillation amplitude sufficiently high as to prevent entrapment. Tapping ModeTM can also be used in a fluid environment. Fluid Tapping ModeTM is an ideal environment for biological samples where desiccation can alter

or destroy the surface being studied. In some cases the fluid can even be advantageous, by dampening environmental vibrations that induce artifacts into the image.

Chapter 3

Investigation of Mussel Adhesive Protein Adsorption on Polystyrene and Poly(octadecyl methacrylate) Using Angle Dependent XPS, ATR-FTIR and AFM

(All ATR FT-IR experiments presented in this chapter were carried out by Dr. Peter Suci)

3.1 Introduction

In the marine environment, microorganisms adhere tenaciously to virtually every known solid surface. Despite many years of research effort, the molecular interactions that are responsible for microbial adhesion and fouling of surfaces remain obscure. An understanding of these interactions would contribute to the development of surfaces that resist colonization of microorganisms. One reason why the molecular interactions are not understood is because microbial adhesion to surfaces is a multifactorial process that involves many types of bonding (McEldowney and Fletcher, 1986). To further complicate the situation, it has been shown that prior to microbial adhesion, a proteinaceous conditioning film forms on the surface of the substratum (Baier, 1980). This conditioning film imparts a uniform net negative charge to the surface and masks the substratum properties (Marshall, 1992). The microorganisms attach to the conditioning film through adhesive structures composed of proteinaceous and exopolysaccharide molecules. Therefore, any serious study of microbial adhesion to submerged surfaces must include the characterization of molecular interactions with the conditioning film. Since the conditioning film which forms on the surface in the marine environment is still poorly defined,

simplification of its composition is essential in order to provide a degree of control that will enable the interactions at these surfaces to be characterized. The goal of this research is to characterize the interactions these proteins have when they are associated with two polymer surfaces displaying different functionalities, as is illustrated in Figure 6.

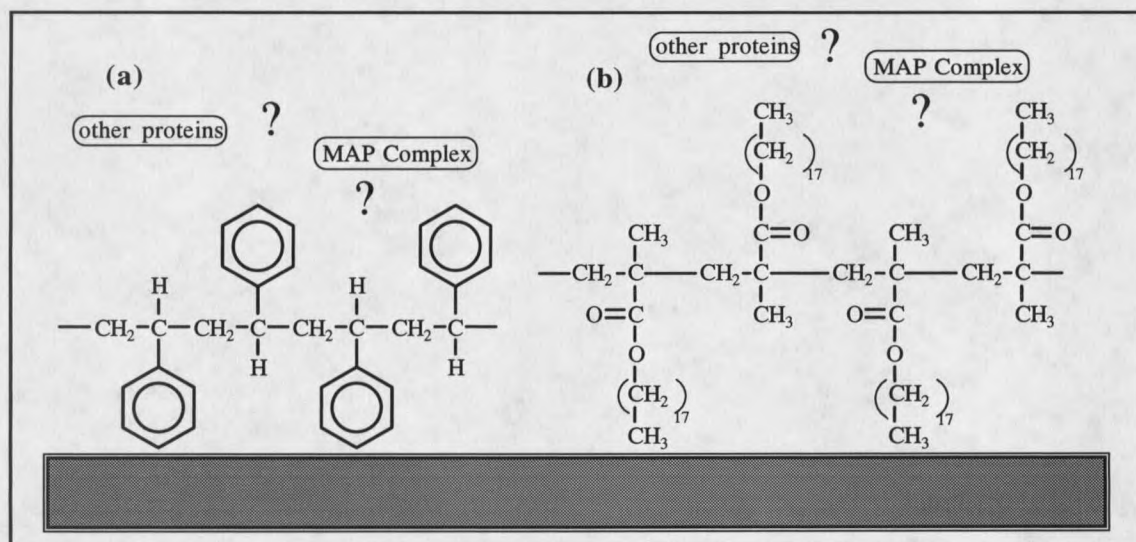


Figure 6. Schematic diagram of the interactions being investigated between the MAP proteins (MeFP-1 and MeFP-2) and between the protein and polymer surfaces of (a) polystyrene and (b) poly(octadecyl methacrylate).

The mussel adhesive proteins (MAP), which contain *Mytilus edulis* foot proteins one and two (MeFP-1 and MeFP-2), were used as a model protein conditioning film. MeFP-1 and MeFP-2 have a highly conserved repeat pattern. The MeFP-1 protein has a well characterized structure consisting of repeating hexa- and decapeptide motifs and has an open conformation with very little secondary structure (Waite, Housley and Tanzer, 1985; Taylor, Ross, Shabanowitz, Hunt and Waite, 1994). These qualities make this protein an ideal model conditioning film since the open conformation and repeat pattern makes the functional groups fully accessible for surface interactions. MeFP-1 and MeFP-2 also have novel compositions, with elevated levels of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and

4- and 3- mono and di-transhydroxyproline (Hyp). These functional groups may confer an adhesive character to the proteins by enabling interactions using quinone redox chemistry (Vogler, 1982; Matsuda, Osaki, Nitta, 1958). However, these protein-surface interactions have yet to be demonstrated. Characterization of the specific protein-surface interactions is a prerequisite to the understanding of microbial attachment and fouling of surfaces.

3.2 Materials

3.2.1 Adsorbates, Solvents and Substrates

Purified MAP from *Mytilus edulis* was obtained from Swedish Bioscience Laboratory (Floda, Sweden) and stored desiccated at -40°C. The amino acid composition according to the supplier is (per 1000 residues): 83 Asp, 74 Thr, 97 Ser, 64 Glu, 69 Pro, 132 Gly, 68 Ala, 50 Val, 25 Ile, 29 Leu, 30 Tyr, 12 Phe, 27 His, 115 Lys, 41 Arg, 41 Hyp and 70 3,4-dihydroxy-L-phenylalanine (L-DOPA). Acetic acid-urea polyacrylamide gel electrophoresis (PAGE) indicated that the MAP preparation consisted of 80% of the two L-DOPA containing proteins, MeFP-1 and MeFP-2, in equal quantities. Two non-L-DOPA containing proteins contributed 20% of this preparation. The protocol for the PAGE was performed using previously described methods and the identification of MeFP-1 (130kD) and MeFP-2 (45kD) were made according to previously published results (Waite and Benedict, 1984). Both MeFP-1 and MeFP-2 contain the unusual catecholic functionality L-DOPA.

Dichloromethylsilane (Aldrich 97%) was used as received. Hexadecane (Aldrich 99+%) was purified by passage through Super I Basic Alumina (Fisher Scientific) five times. All solvents including, chloroform, ethanol and toluene (Aldrich) were HPLC grade.

Optically smooth germanium(111) wafers (Exotic materials Inc., Costa Mesa, CA), 2.54 cm in diameter and 1 mm thick, were cut into 1 cm x 1 cm pieces using a

diamond tipped stylus and spin coated with either polystyrene (PS) or poly(octadecyl methacrylate) (POMA) for the x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) studies. For the attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) studies cylindrical germanium internal reflection elements (IRE's) (Spectra Tech, Stamford, CT) were used.

PS (Aldrich Secondary Standard) was prepared as a 1.5%(W/V) solution in toluene. POMA (Aldrich) was prepared as a 1.5%(V/V) solution in toluene.

3.3 Preparation Of Substratum

3.3.1 *Ge Cleaning Procedure*

The germanium crystals were immersed in an ultrasonic bath of a cleaning solution that was a mixture of isopropyl alcohol saturated with potassium hydroxide for 10 min. The crystals were then removed and immediately placed in an ultrasonic bath of ultra pure water. They were then gently scrubbed with undiluted Micro™ cleaning solution using cotton swabs. The crystals were rinsed in a hard stream of ultra pure water and immersed in a series of ultrasonic solvent baths, for 5 min each in; ultra pure water (twice), ethanol, and chloroform. The crystals were then immediately dried under a stream of hydrocarbon free, dry nitrogen and transferred to an inert atmosphere (dry N₂) chamber, prior to silanization. The above cleaning procedure was performed immediately before the silanization reaction to prevent any adsorbed materials from contaminating the surface.

Auger electron spectroscopy, using a Phi Model 595 scanning Auger microprobe, indicated that the composition of the outermost surface region of the germanium substratum, when cleaned by this protocol, was $9.1 \pm 1.4\%$ carbon, $6.5 \pm 2.0\%$ oxygen, and $83.9 \pm 2.2\%$ germanium.

3.3.2 Silanization Procedure

The germanium crystals were silanized with dichloromethylsilane to improve the adhesion of the polymer films and to prevent film delamination in aqueous solution. All silane monolayers were prepared under an inert atmosphere of dry N_2 . Monolayers of dichloromethylsilane were formed by immersing clean Ge crystals into a freshly prepared solution of dichloromethylsilane in n-hexadecane. All glassware used was cleaned with "piranha" solution, consisting of a 70:30 mix of concentrated H_2SO_4 and 30% H_2O_2 respectively. [WARNING: Piranha solution reacts violently, even explosively with organic materials (Dobbs, Bergman and Theopold, 1990).] Individual solutions were prepared by mixing a solution that is 5×10^{-3} M dichloromethylsilane in n-hexadecane. Each solution was stirred for 5 min before a germanium crystal was introduced. The reaction vessel was then capped and stored at room temperature for 12 h. Upon removal from the silane solution, the crystals were immediately rinsed with 50 ml of chloroform. They were then removed from the inert atmosphere and extracted in a sohxlet extractor with hot chloroform for 30 min to remove any excess silane. They were then cured in an oven at $112^\circ C$ for 3 h. Contact angle measurements were performed with water to estimate the quality of the silane film. Surfaces with contact angles less than 100° proved inadequate for polymer adhesion and were discarded.

3.3.3 Preparation Of Polymer Surfaces

PS and POMA polymer films were spin cast onto presilanized, germanium fragments for the XPS and AFM studies. PS was used as a 1.5% (W/V) solution in toluene and POMA was used as a 1.5% (V/V) solution in toluene. Pre-silanized germanium crystals were completely covered with polymer solution and then immediately spin cast at 3500 rpm for 2 min. The polymer films were dried at room temperature for 24 h.

For the ATR-FTIR studies the cylindrical germanium IRE's were silanized as described above. PS and POMA were dip coated onto germanium IREs at a speed of 0.5 cm/s.

X-ray photoelectron spectroscopy (Surface Science Instruments, Model SSX-100, 600 mm diameter spot size, monochromatized aluminum Ka source) of the polymer films, indicated that the films were continuous (i.e., no germanium or silane was detected). AFM Images acquired in Fluid Tapping™ mode and cold-probe XPS analysis indicate that the spin cast polymer surfaces are stable when hydrated.

3.3.4 Protein Adsorption Protocol

For the XPS and AFM studies the polymer coated germanium substrata were placed in a glass flow cell with entrance and exit tubing ports to allow for protein adsorption and subsequent rinse. For ATR-FTIR adsorption experiments, the polymer coated germanium IRE's were placed within a stainless steel flow chamber (Circle Cell™, Spectra Tech, Stamford, CT). Fluid was introduced and displaced through entrance and exit ports at each end of the Circle Cell™.

All protein films were deposited onto freshly prepared polymer surfaces after the 24 h drying period. A stock solution of 1 mg/ml MAP was prepared in dilute HCl (pH 2.5) with deionized double distilled water, deaerated with N₂. The stock solution was stored at 5°C. A 50 µl aliquot of this solution was added to 0.45 ml of dilute HCl (pH 2.5). For the XPS and AFM studies this mixture was delivered into the flow cell containing the substratum that was to undergo protein adhesion. MAP was allowed to adsorb by raising the pH to 8.0 by delivering a 0.5 ml aliquot of a pH 10.9 solution into the reaction chamber, bringing the concentration of the protein to 50 µg/ml. For the ATR-FTIR studies a 50 µg/ml MAP solution was prepared as above before delivery into the flow cell. After a 1 h adsorption, the substratum was rinsed of any unadsorbed protein by flowing an

aqueous solution at pH 8.0 through the reaction chamber at a rate of 100 ml/min for 3 min. The ATR-FTIR Circle CellTM was rinsed at a rate of 0.5 ml/min for 60 min. The samples were removed and dried in hydrocarbon free dry air overnight.

3.4 Surface Characterization

3.4.1 Atomic Force Microscopy Imaging

All surfaces were imaged using a Nanoscope III AFM (Digital Instruments, Inc. Santa Barbara, CA) with a 350D scanner and a 3-101 optical head. The instrument was used in contact mode using square pyramid microfabricated silicon nitride cantilevers which were 100 μm in length and had a spring constant of 0.38 N/m. The images were recorded using 1 μm $\times$ 1 μm and 5 μm $\times$ 5 μm scan areas at 512 scans per area with a scan rate of 2 s^{-1} . All images were acquired in air and were stable with time and reproducible.

3.4.2 Angle Resolved X-Ray Photoelectron Spectroscopy

XPS spectra were obtained from a Surface Science Instrument Model SSX-100 spectrometer. A 5 eV flood gun was used to offset charge accumulation on the samples. A 600 μm diameter spot size was scanned using a monochromatized Aluminum K α x-ray source at 350 Watts and pass energies between 25.0 eV (resolution 1) and 150 eV (resolution 4). Elemental composition was calculated on peak areas from the C1s, N1s, and O1s core levels. Relative peak areas were calculated by fitting the high resolution C1s, N1s and O1s peaks with Gaussian functions. Before the variable angle study was conducted an initial survey at 80° (from the surface) was completed. Depth profiles were performed using variable angle XPS data collected at take off angles of 10°, 22°, 35° and 80°. The elemental compositions at the initial 80° survey were compared with the final 80° angle study to ensure no x-ray damage had occurred during analysis. The data were collected with the wide angle acceptance lens masked with a 12° slit. The binding energy

scale was referenced by setting the CH_x peak maximum in the C1s spectrum to 285.0 eV (Ratner and Castner, 1994).

3.4.3 ATR-FTIR Spectrometry

The time course of MAP adsorption in a hydrated state was followed by ATR-FTIR spectrometry. During the time course of each experiment infrared (IR) spectra were acquired every 5 min. A Perkin Elmer Model 1800 Fourier transform infrared (FT-IR) spectrophotometer equipped with a liquid N₂ cooled, medium range mercury-cadmium-telluride detector (5000-580 cm⁻¹) was used to collect the ATR-FTIR spectra. Interferograms were double-sided, apodization was a weak Beer-Norton function, the range was 4000-700 cm⁻¹ with an interval of 1 cm⁻¹ and nominal resolution of 2 cm⁻¹; 50 interferograms were averaged per spectrum; water vapor bands were removed by subtraction of a pure water vapor spectrum; fluctuations in intensity of the strong water band at 1640 cm⁻¹ resulted in appearance of this band in the difference spectra. This residual water absorption band was removed by subtracting out a pure water spectrum using the ratio of areas of the absorption water band centered at 2120 cm⁻¹ as a normalization factor (Dousseau, Therien and Pezolet, 1989). Variation in absorbance values resulting from slight differences in alignment of the flow chamber on the optical bench and coating with polymer films were normalized by using the area of the water absorption band at 1640 cm⁻¹ (area: 1540 cm⁻¹ to 1740 cm⁻¹) as an internal standard (Fink, Hutson, Chittur and Gendreau, 1987). Areas of spectral features were computed for the region bounded by the data curve and a linear baseline drawn between the two end points of the integration.

Protein surface coverage was estimated based on area of the amide II band using published correlations. Adsorption conditions of Fink et al. (Fink, Hutson, Chittur and Gendreau, 1987) resemble approximately those here (saline solution, pH 7.4 on

germanium). Extinction coefficients for solution phase bovine serum albumin compare favorably with our estimates (within 80%). Fink et al. (Fink, Hutson, Chittur and Gendreau, 1987) obtained correlations for adsorbed human albumin, immunoglobulin and fibrinogen. Using their data, a factor for conversion of amide II areas to surface coverage in $\mu\text{g}/\text{cm}^2$ can be estimated. This conversion factor is $0.26 \pm 0.12 \mu\text{g}/\text{cm}^2$ per unit area amide II ($\text{abs}\cdot\text{cm}^{-1}$).

3.5 Results

3.5.1 Atomic Force Microscopy Imaging

MAP adsorbed to clean PS and POMA from a solution with a bulk protein concentration of $50 \mu\text{g}/\text{ml}$ was imaged using AFM. Figure 7 shows AFM contour images of $1 \mu\text{m} \times 1 \mu\text{m}$ areas of the two substrata before MAP adsorption. Figure 8 and 9 shows AFM contour images of $1 \mu\text{m} \times 1 \mu\text{m}$ (a) and $5 \mu\text{m} \times 5 \mu\text{m}$ (b) areas after MAP adsorption to PS and POMA, respectively. Before MAP adsorption, the polymer surfaces are extremely smooth with almost no surface features and RMS surface roughness values of 1.035 nm and 0.546 nm for PS and POMA respectively.

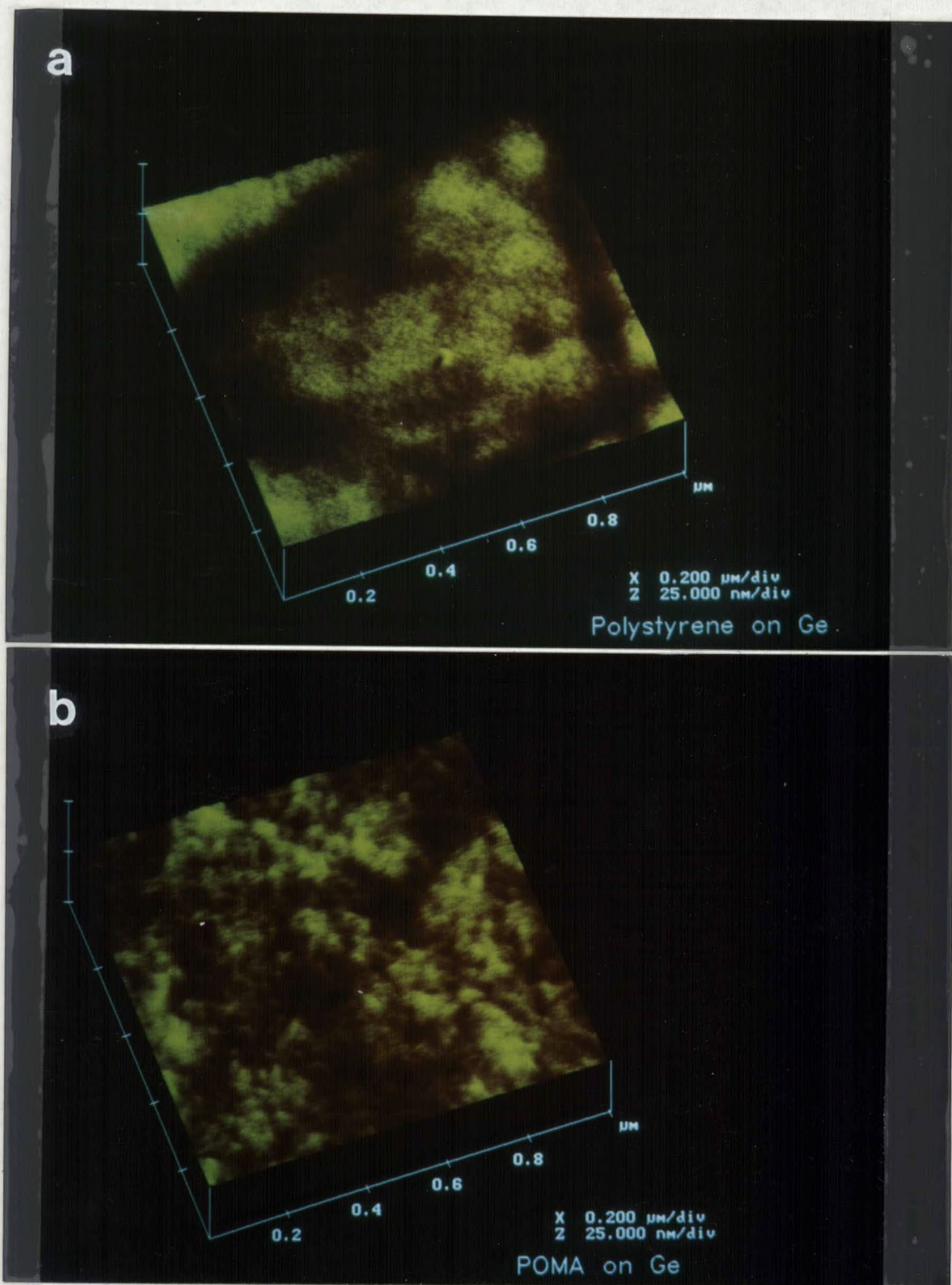


Figure 7. AFM contour image of; 1 μm x 1 μm area of PS (a) and POMA (b) before protein adsorption.

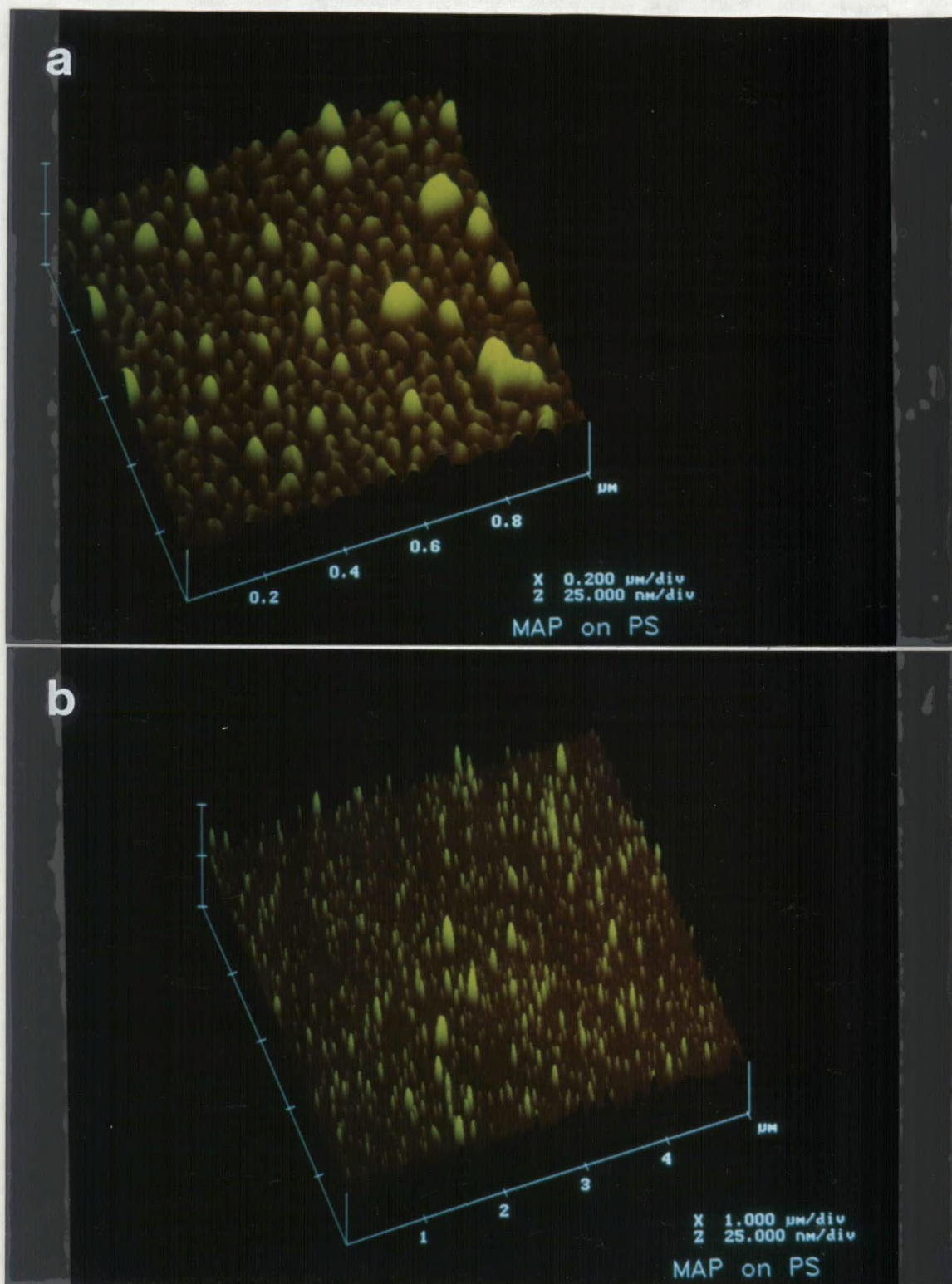


Figure 8. AFM contour images of; 1 $\mu\text{m} \times 1 \mu\text{m}$ area of MAP adsorbed to PS (a) and 5 $\mu\text{m} \times 5 \mu\text{m}$ area of MAP adsorbed to PS(b).

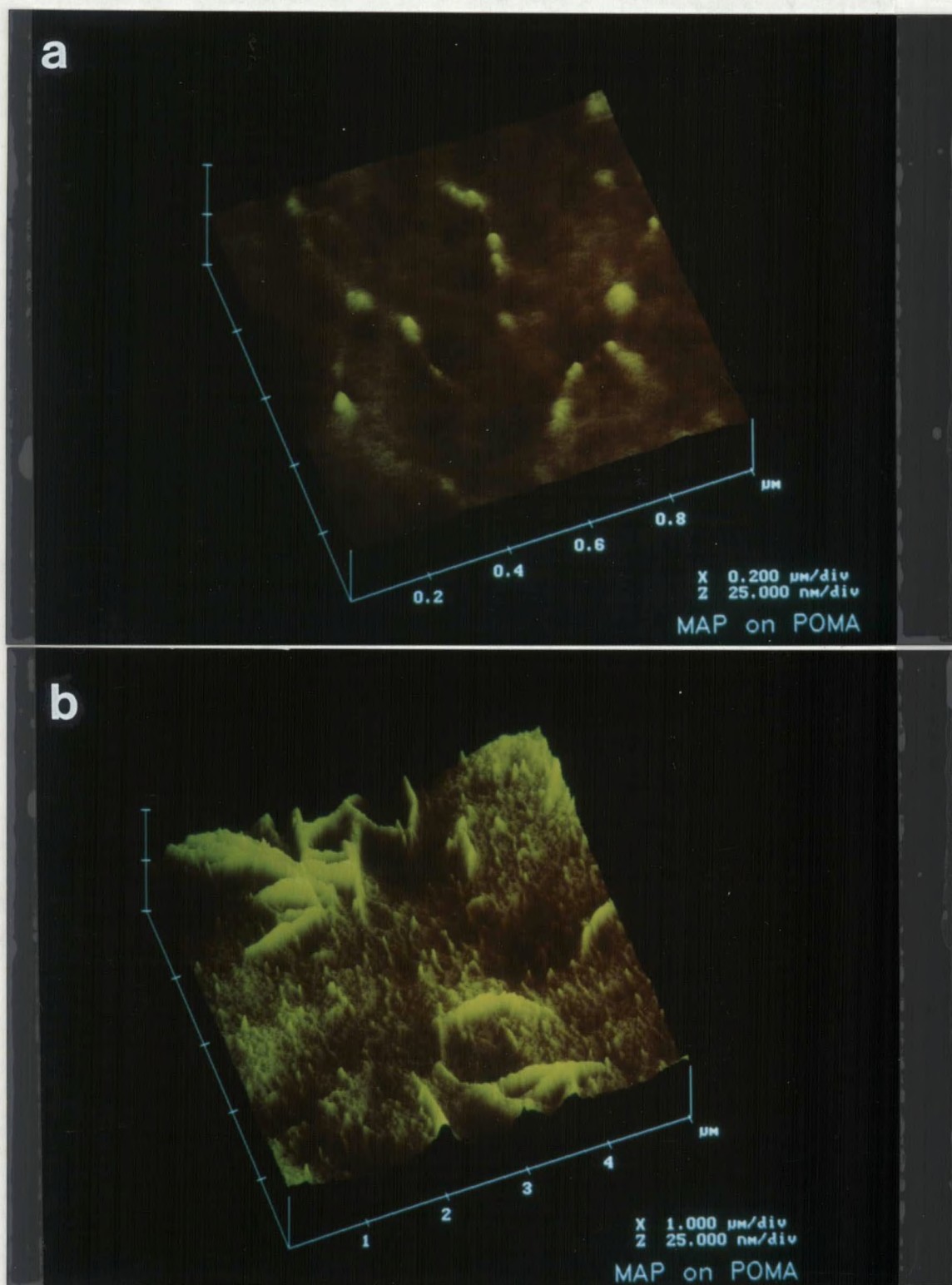


Figure 9. AFM contour images of; 1 $\mu\text{m} \times 1 \mu\text{m}$ area of MAP adsorbed to POMA (a) and 5 $\mu\text{m} \times 5 \mu\text{m}$ area of MAP adsorbed to POMA (b).

Adsorption of MAP to the PS surface resulted in the formation of closely packed, repeating structures as is shown in Figure 8a. Cross sectional analysis of these features shows an average height of 9.48 ± 3.05 nm and a width of 33.02 ± 7.82 nm. The $5 \mu\text{m} \times 5 \mu\text{m}$ scan area, shown in Figure 8b, reveals that the features observed at high magnification cover larger areas of the surface, suggesting that the surfaces are homogeneous and have continuous protein coverage. In contrast, MAP adsorbed to the POMA surface displays very different protein features that appear to be linearly ordered as revealed in the $1 \mu\text{m} \times 1 \mu\text{m}$ scan area in Figure 9a. Cross-sectional analysis reveals that these features have an average height of 2.45 ± 1.35 nm and a width of 68.4 ± 3.91 nm. The $5 \mu\text{m} \times 5 \mu\text{m}$ scan area, shown in Figure 9b, shows the protein features on this surface to be very heterogeneous, with larger more complex fibrous structures. These features extend 19.36 nm high and 179.69 nm in width, with some as long as $2.4 \mu\text{m}$. Because of the heterogeneous nature of MAP on this surface it is difficult to determine whether the surface coverage of the protein is continuous based on this technique.

3.5.2 Angle Resolved X-Ray Photoelectron Spectroscopy

Angle dependent XPS of the surfaces studied in Figures 8 and 9 further reveals the differences in MAP adsorption to PS and POMA surfaces. A detailed examination of the C1s region of clean PS and POMA are given in figure 10. MAP adsorbed to PS and POMA at take off angles of 80° and 10° are given in Figure 11 and 12, respectively.

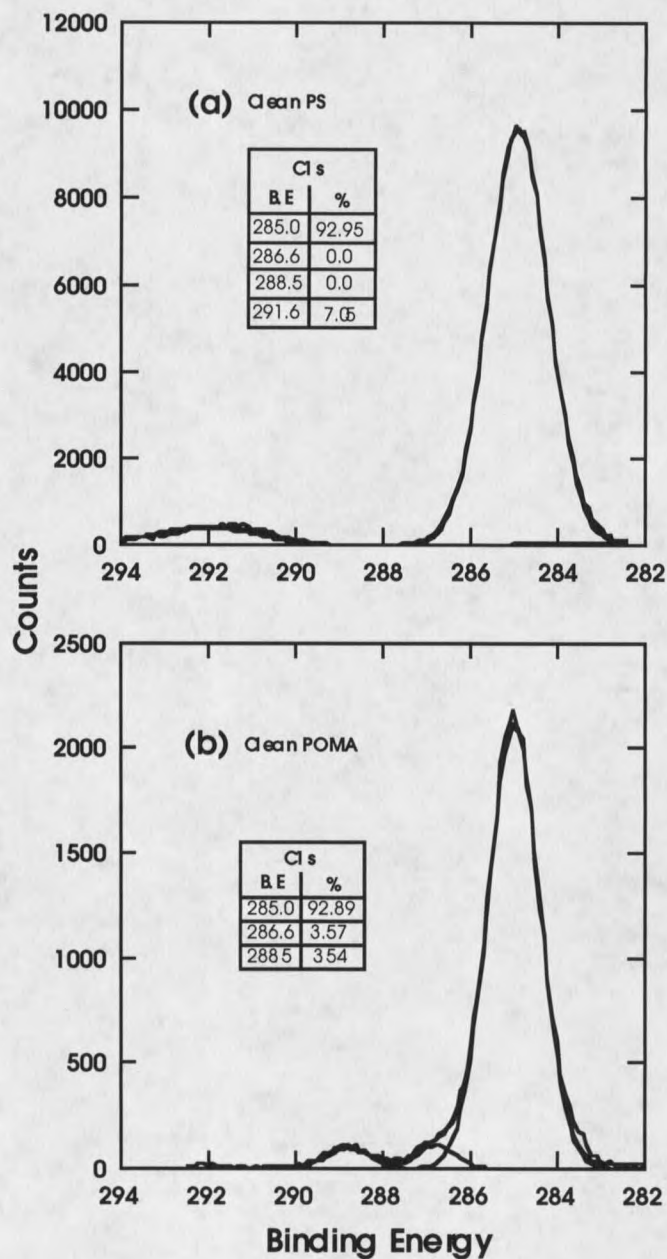


Figure 10. C1s spectra of clean PS (a) and POMA (b).

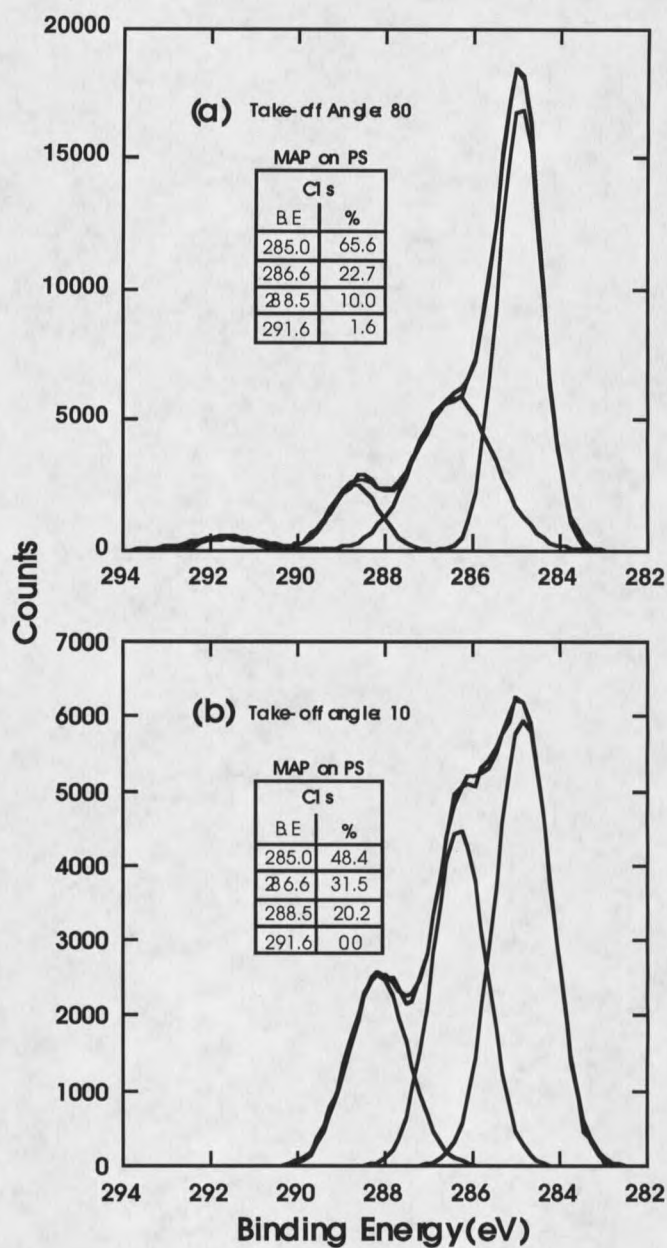


Figure 11. C1s spectra of MAP adsorbed to PS at take-off angles of 80° (a) and 10° (b).

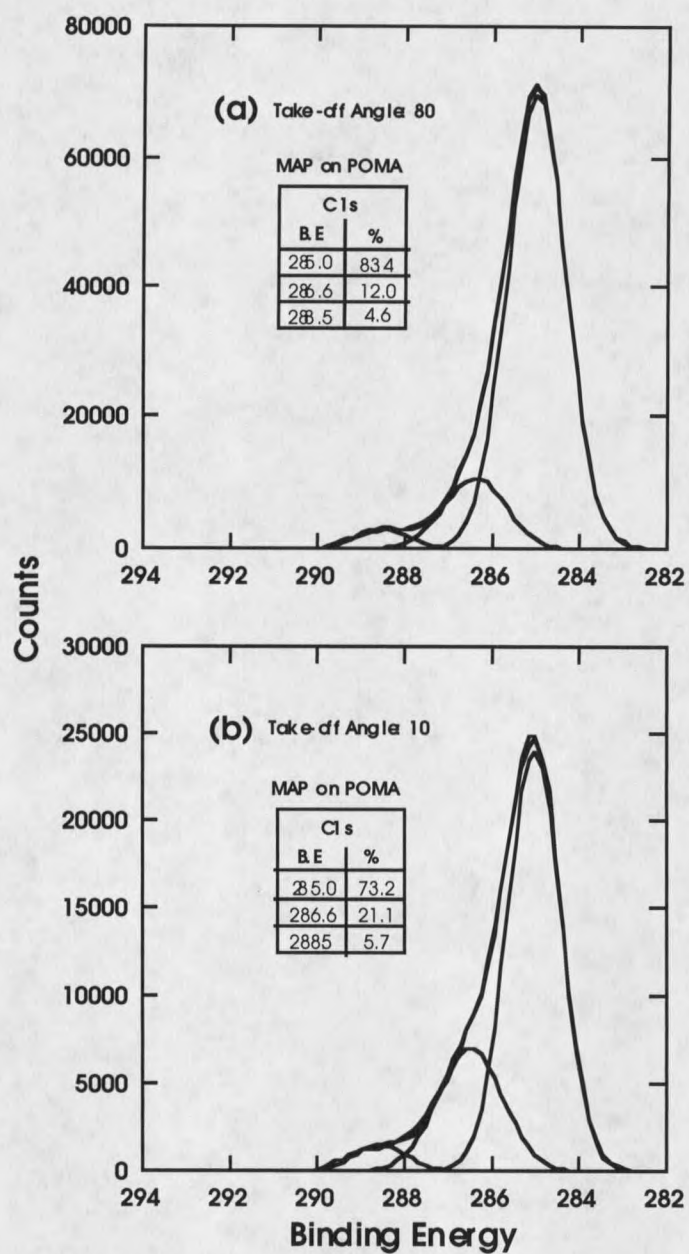


Figure 12. C1s spectra of MAP adsorbed to POMA at take-off angles of 80° (a) and 10° (b).

Each peak is contributed by different chemical groups. The C1s peak at a binding energy of 291.6 eV is attributed to the π - π^* transition in the aromatic rings of the polystyrene. The peak at 288.5 eV arises from the N-C=O and O-C=O functionalities on the protein and in the methacrylate chain, respectively. The C1s peak at a binding energy of 286.6 eV is attributed to the C-N and C-O functionalities in the protein and the C-O-C functionality of the POMA. The dominant peak in both sets of spectra is at 285.0 eV. This peak originates from the aliphatic carbon in both the polymers and the protein.

Comparing the C1s regions of MAP adsorbed to PS at 80° and 10° take off angles, reveals a decrease in the aliphatic peak at 285.0 eV and disappearance of the peak associated with the π - π^* transition. The disappearance of the π - π^* transition and the decrease in the aliphatic peak relative to the 286.6 eV and 288.5 eV peaks from the protein indicates that at a take off angle of 10° the signal from the PS has disappeared from the spectra and signal intensity arises only from the protein. The C1s region of MAP adsorbed to POMA reveals an increase in the peak at 286.6 eV relative to the 285.0 eV peak at the lower take off angle. This also indicates a greater contribution from the protein to this C1s spectra.

The elemental compositions at the different angles were used with the calculated escape depths for each angle to gain insight on the elemental depth distribution of the proteins adsorbed to the polymer surfaces. The escape depths were calculated using parameters for organic compounds to calculate the inelastic mean free path and subsequently the escape depth using equations previously defined (Seah and Dench, 1980). Table 3 indicates that nitrogen is increasing with depth when MAP is adsorbed to PS, whereas, this trend is reversed on POMA.

Table 3: Atomic Concentration of MAP Adsorbed to PS and POMA.

*Mean Atomic Percent					
<u>Sample</u>	<u>Take-off Angle</u>	<u>Escape Depth</u>	<u>C</u>	<u>O</u>	<u>N</u>
MAP	80	84.3	78.8±6.3	13.5±4.4	6.2±3.2
Adsorbed to	35	49.1	76.5±7.5	15.8±3.5	7.5±3.6
Polystyrene	22	32.1	74.3±8.4	16.0±4.8	8.5±4.3
	10	14.9	72.8±8.5	16.7±4.6	8.9±4.2
MAP	80	84.3	90.6±1.5	6.2±1.6	1.5±0.1
Adsorbed to	35	49.1	92.1±0.6	6.6±0.3	1.0±0.1
POMA	22	32.1	92.7±0.4	6.5±0.1	0.6±0.1
	10	14.9	94.8±0.6	4.7±0.9	0.5±0.5

.....*Taken from three separately prepared surfaces

This indicates that MAP adsorbed to the polystyrene surface shows a nitrogen distribution that is enriched at the surface of the adsorbed protein film. In contrast, the MAP adsorbed to the POMA surface displays a depth profile that shows the nitrogen enrichment from the adsorbed protein at a maximum at the polymer surface.

3.5.3 ATR-FTIR Spectrometry

Figure 4a, and 4b shows ATR-FTIR spectra of MAP adsorbed onto PS and POMA polymer films after the 60 minute rinse period. The time course of adsorption/desorption was followed based on the areas (1591 cm^{-1} to 1493 cm^{-1}) of the amide II band (indicated by iii in Figures 4a and 4b). This is shown in Figure 4c for each of the two surfaces (PS and POMA). Adsorption appears to be irreversible. The surface coverage of MAP at the end of the rinse period on PS and POMA surfaces, estimated from the correlations specified in the methods section, is 0.045 ± 0.021 and $0.066\pm0.031\text{ }\mu\text{g}/\text{cm}^2$, respectively.

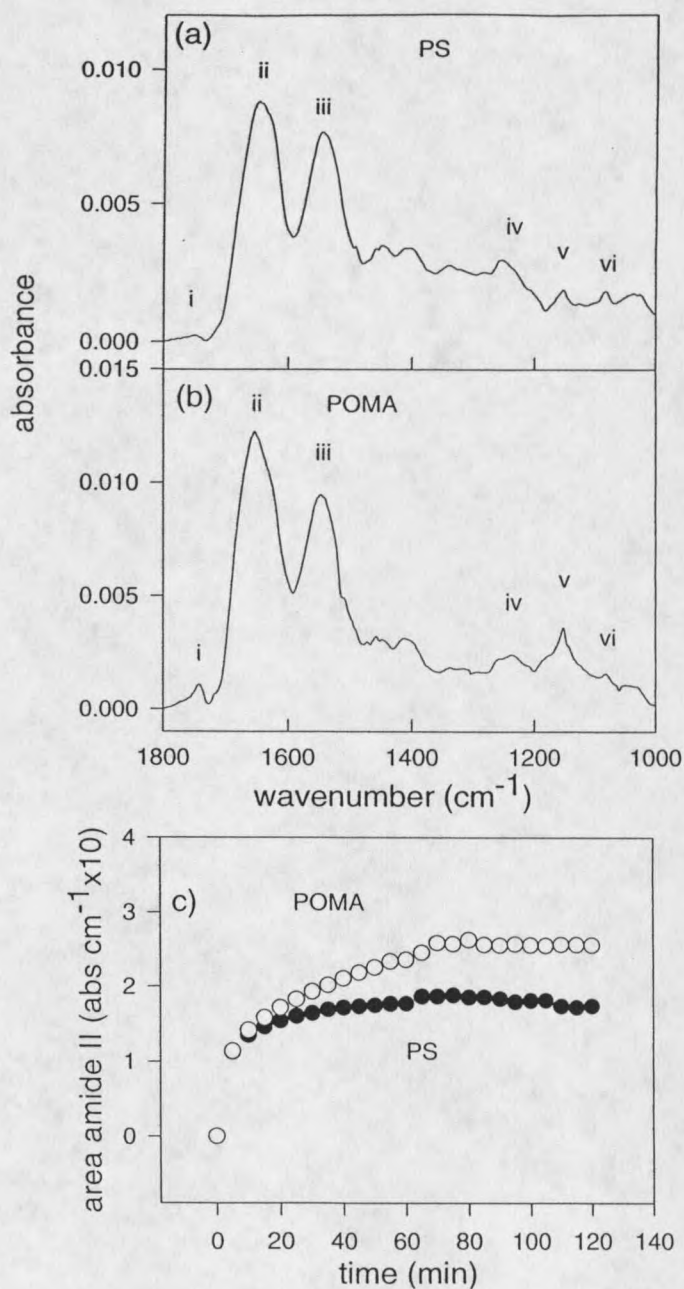


Figure 13. ATR-FTIR spectra of adsorbed MAP to: PS (a) and POMA (b). Roman numerals indicate spectral features discussed in the text. (c) time course of adsorption (60min.) and desorption (60min.) followed by the area of the amide II band (iii). Open circles represent PS and closed circles POMA.

Comparison of the spectra presented in Figures 4a and 4b reveals a number of differences in spectral features. (Features are indicated in Figures 4a and 4b by lower case Roman numerals). A band centered at 1740 cm^{-1} (i) is evident in MAP on POMA which does not appear in the spectrum of MAP on PS (although there is a slight band centered at 1730 cm^{-1} in this spectrum). The amide I band (ii) is centered at 1645 cm^{-1} and 1654 cm^{-1} in the MAP on PS and POMA, respectively. Spectral features in the region from 1300 cm^{-1} to 1200 cm^{-1} (iv) are typically attributed to amide III vibrations which are sensitive to protein secondary structure (14,15,16) and less obscured by the large water band centered at 1640 cm^{-1} than the amide I and II bands. In this region the spectrum of MAP on PS and POMA differ significantly. Distinct features centered at 1150 cm^{-1} (v) and 1083 cm^{-1} (vi) in both spectra are unusual for adsorbed proteins. The band centered at 1150 cm^{-1} is especially prominent in the MAP on POMA.

3.6 Discussion

The results presented here, demonstrate that the functional groups that are present at a polymer surface can influence protein-protein and protein-surface interactions. The protein surface interactions that can occur in the polymer-protein systems studied here are limited to two categories: one surface capable of undergoing MAP-surface pi-pi bond overlap interactions (PS) and one with no favorably energetic MAP-surface interactions (POMA). The PS surface provides a hydrophobic surface with an aromatic character and a medium surface free energy, and the POMA surface provides a hydrophobic low energy surface with an aliphatic functionality.

There are four mechanisms that have been proposed to play important roles in MAP-MAP and MAP-surface interactions: hydrogen bonding, metal-ligand complexes, Michael-type addition compounds derived from o-quinones, and charge transfer complexes (Waite, 1987). Olivieri (Olivieri, Loomis and Baier, 1992) has collected data that suggests

that MAP can orient itself towards oxide surfaces enabling the L-DOPA residues to interact with the surface through hydrogen bonding. Hansen (Hansen, Luther and Waite, 1994) has recently found that MAP interacts with stainless steel by complexing and binding with surface metals. The Michael-type addition compounds are driven by the catechol oxidase enzyme that is co-secreted with the proteins in the natural system (Waite, 1989). In the system studied here, there are no divalent cations or metal ions to provide metal-ligand complexation. There are no enzyme driven reactions because the catechol oxidase does not survive the purification procedures for the MAP proteins and there are no functionalities present on the surface to allow hydrogen bonding interactions. However, at an elevated pH of 8 (approximately that of sea water and the pH during adsorption) the catechol functionality on the L-DOPA can undergo a spontaneous reverse dismutation to the o-quinone that is capable of interacting through a quinhydrone charge-transfer complex, illustrated in Figure 14.

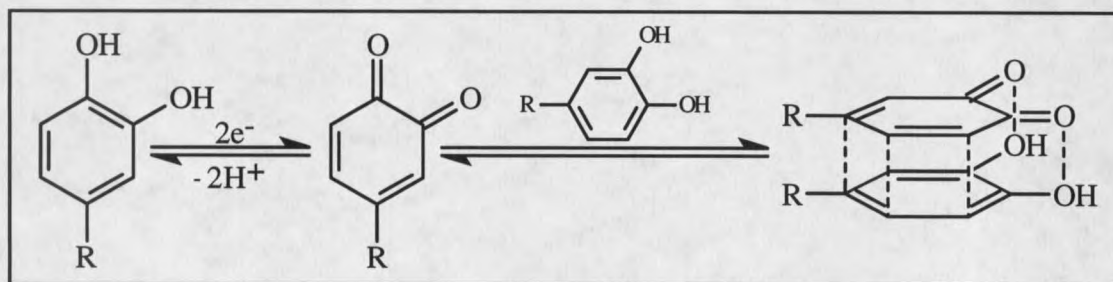


Figure 14. Quinhydrone-charge transfer complex of the catechol functionality of MAP.

The PS surface that displays an aromatic functionality would inhibit this MAP-MAP interaction because of the ability of PS to undergo pi-pi overlap interactions with the aromatic functionality of PS and the aromatic side chains of the MAP. The resulting surface topography after MAP adsorption to PS reveals homogeneous, repeating structures that would suggest this type of MAP-surface interaction. Furthermore, the dimensions of

these features on the PS surface, are representative of individual MeFP-1 and MeFP-2 molecules (Rzepecki, Hansen and Waite, 1992). In order to maximize the pi-pi interactions with the surface, the MAP will orient the aromatic side chains facing the polymer surface. This reasoning is supported by the decreasing nitrogen composition with depth in the XPS data. Since there are no energetically favorable MAP interactions with the POMA surface, the catecholic functional groups of the adsorbed protein are free to interact with each other and form charge-transfer complexes, as shown in Figure 14. In this case the adsorption of the protein on a surface that appears to have no favorably energetic mechanisms for interactions is most likely driven by van der Waals forces, arising from the cross-linked protein with the surface. This results in the aggregated protein structures and the apparent linear order of the protein shown in the AFM images on this polymer surface. Furthermore, the smaller linear features on the POMA surface are representative of cross-linked MeFP-1 and MeFP-2 on the surface (Rzepecki, Hansen and Waite, 1992). The larger, fibrous structures on the POMA could arise from aggregated or cross-linked MAP. In either case, the AFM images suggest that the differences in surface chemistry on these two polymer surfaces strongly influence protein adsorption. Recent images obtained under fully hydrated conditions by AFM in Tapping ModeTM suggest that dehydration is not responsible for the gross differences observed by MAP on these surfaces.

When MAP adsorption to PS and POMA was evaluated under hydrating conditions by ATR-FTIR (Baty, Suci, Tyler and Geesey, 1995), spectral differences further suggested different protein interactions with the chemically distinct polymers. Differences in IR spectral features in the amide III region of proteins have been attributed to differences in secondary structure (Kaiden, Matsui, Tanaka, 1987; Jakobsen, Wasacz, 1987; Pitt, Spiegelberg and Cooper, 1987). Although, it is difficult to make specific assignments unless the proteins consist entirely of one domain, differences observed in this amide III region ($1200\text{-}1300\text{ cm}^{-1}$) do indicate a difference in the hydrogen bonding pattern between

the amide linkages of MAP on the PS and POMA surface. Therefore, the IR results are consistent with the XPS and AFM data which indicate that MAP organization and/or orientation are different on the PS and POMA surfaces. Of the three methodologies used, ATR-FTIR has the least spatial resolution. Since these spectral differences represent an average over the entire IRE surface (approx. 2.5 cm^2) it is unlikely that the phenomenon is confined to a small region of the surface.

The bands centered at 1150 cm^{-1} and 1083 cm^{-1} may originate from residues whose repetitive motif results in resonance summation at particular frequencies, making them visible above the background. An attempt to identify these bands as arising from specific residues by comparison with ATR-FTIR spectra of aqueous solutions of various compounds has so far been unsuccessful. Nevertheless, there is a resemblance between the contrasting spectral features which appear for MAP on PS and POMA and those reported for adsorption of the blood plasma protein, fibronectin, on a series of functionalized polyurethanes (Jakobsen, Wasacz, 1987). Notably, differences were observed in the amide III and I regions, and a band appeared to varying degrees in the $1720\text{-}1740 \text{ cm}^{-1}$ region. The latter band arises from the carbonyl group, and appears when a carboxylate functionality is protonated (i.e., salt to acid form) (Olivieri, Loomis, Meyer and Baier, 1990). For fibronectin, the appearance of this band was most prominent for the polyurethanes having the most hydrophobic functionalities. It was hypothesized that the acidic residues of the adsorbed protein entered a region of low dielectric constant proximal to the hydrophobic surface, shifting the equilibrium toward the protonated form. The MAP protein MeFP-2 is rich in acidic residues and it is speculated that it may mediate bridging between MeFP-1 (Rzepecki, Hansen and Waite, 1992). It is possible that the highly interconnecting pattern observed in the AFM images of MAP adsorbed to POMA results from this crosslinking reaction shown in Figure 13, of MeFP-1 with MeFP-2.

Some degree of caution is necessary in interpreting the spectral features in the region from 1200 to 1300 cm^{-1} as arising purely from the amide III resonance frequencies. Both L-DOPA and tyrosine exhibit bands in this region. In fact, the band at approximately 1250 cm^{-1} has been previously attributed to the L-DOPA residues (Olivieri, Loomis and Baier, 1992; Olivieri, Loomis, Meyer and Baier, 1990). Therefore, the differences in this region for MAP on PS and POMA may indicate differences in the interaction between these residues and the polymer functional groups. These differences in protein-surface interactions between an aromatic surface (PS) and an aliphatic surface (POMA) are also indicated by the contrasting shape of the bands centered at 1150 cm^{-1} and 1083 cm^{-1} .

The research presented here demonstrates that the functional groups that are present on the PS and POMA polymer surfaces will influence MAP-MAP and MAP-surface interactions. The XPS, AFM and ATR-FTIR data demonstrates that differences in surface interactions can be correlated through these complementary analytical techniques.

Chapter 4

The Adsorption of Adhesive Proteins From the Marine Mussel, *Mytilus edulis*, on Polymer Films in the Hydrated State Using Angle Dependent XPS, and AFM

4.1 Introduction

The Marine mussel, *Mytilus edulis*, produces a series of adhesive proteins that allow the organism to attach to a variety of surfaces in an underwater environment (Waite, 1987). These proteins serve to support and bind components of the adhesive holdfast composed of byssal threads (Waite 1983). The byssal threads are comprised of a collagenous matrix that is bound with a series of phenolic proteins. There have been four proteins identified in the byssal threads that are thought to serve these structural and adhesive functions: *Mytilus edulis* Foot Proteins (MeFP), 1, 2, 3 and 4, collectively termed Mussel Adhesive Proteins (MAP). It has been suggested that MeFP-1 serves as one of the initial adhesive components as well as a protective varnish on the surface of the byssal thread (Waite, 1983; Benedict and Waite, 1986). It is also believed that MeFP-2 serves a structural function, binding the collagen fibers within the byssal threads (Rzepecki, Hansen and Waite, 1992). MeFP-3 is thought to play a role as an initial surface primer and the function of MeFP-4 is still uncertain (Papov, Diamond, Biemann and Waite, 1995). The ability of *Mytilus edulis* to form a tenacious adhesive bond rapidly at a solid-liquid interface entails more than just depositing these proteins on the surface. Before adhering to a surface the mussel foot first explores and then scrubs the prospective surface by cavitation. The phenolic proteins and collagen are then injected into the already cavitated mussel foot by

phenol and collagen glands forming the plaque that is attached to the surface (Vitellaro-Zuccarello, 1980). Despite the complexities involved attempts to fabricate a biomimetic version of the MAP adhesive are underway. However, commercial uses for MAP have been limited, due to the lack of understanding of how these proteins function as an adhesive.

MAP has seen recent interest in the biomedical community as a tissue adhesive. MAP has been studied for its ability to fix chondrocyte allografts internally (Pitman, Menche, Song, Ben-Yishay, Gilbert, Grande, 1989; Grande and Pitman, 1988). The protein has also been used in experimental epikeroplasty in laboratory animals, and as an adhesive agent to increase cellular attachment to substrata (Robin, Picciano, Kusleika, Salazar and Benedict, 1988; Olivieri, Rittle, Tweden and Loomis, 1992). Recent studies have also indicated that MAP enhances the attachment of osteoblasts and epiphyseal cartilage cells to substrata (Fulkerson, Norton, Gronowicz, Picciano, Massicotte and Nissen, 1990). However, practical application of MAP as a biocompatible adhesive has not yet been realized, as the actual anchoring mechanism is poorly understood. Despite MAP's tenacious adhesive action in the natural environment, this function has not been duplicated with the purified proteins. Although the biochemistry of the adhesive plaque has been extensively and elegantly characterized, relatively little work has been done to characterize the molecular interactions between components and surfaces (Baty, Suci, Tyler and Geesey, 1995; Suci and Geesey, 1995). Therefore, essential design criterion may be lacking. A greater understanding of how MAP binds to surfaces is essential in developing MAP as a useful adhesive.

The goal of this study was to investigate the adsorption of mussel adhesive protein (MAP) to polystyrene (PS) and poly(octadecyl methacrylate) (POMA) surfaces in the hydrated state using cold probe techniques in variable angle x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) using fluid Tapping Mode™.

Surfaces were also dehydrated at room temperature and analyzed using variable angle XPS to assess the contribution that hydration has on the adsorbed protein layer. XPS was used to quantify the elemental composition with depth of the adsorbed protein and AFM was used to provide information about the architecture of the protein adsorption to the polymer surfaces.

4.2 Materials and Methods

4.2.1 Adsorbates, Solvents and Substrates

The MAP used in this study consists of two of the *Mytilus edulis* foot proteins, (MeFP-1 and 2). MeFP-1 is a 130 kD protein consisting of highly conserved tandemly repeated decapeptide sequences (Waite, Housley, and Tanzer, 1985). MeFP-1 has extensive hydroxylation of tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and of proline to hydroxyproline (HYP) (Waite, Housley, and Tanzer, 1985; Waite and Tanzer, 1980). This protein is one of the first proteins described to contain DOPA in its primary sequence and is one of the few proteins found in nature that contains hydroxyprolines in non-collagenous sequences (Taylor, Ross, Shabanowitz, Hunt and Waite, 1994). The hydroxyprolines impart a slight circular dichroism, present as 20% β -turns, due to the hydroxyprolines. MeFP-1 has no other secondary structure (Williams, Marumo, Waite and Henkens, 1989).

The MeFP-2 is a 42-47 kDa protein consisting of at least three repeating motifs. In contrast to MeFP-1, MeFP-2 contains 6-7 mol% of the disulfide containing amino acid cystine, indicating considerable secondary structure. Both MeFP-1 and MeFP-2 have novel compositions, with elevated levels of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and 4- and 3- mono and di-transhydroxyproline (Hyp). These functional groups may confer an adhesive character to the proteins by enabling interactions using quinone redox chemistry

(Vogler, 1982; Matsuda, Osaki and Nitta, 1958). However, these protein-surface interactions have yet to be demonstrated.

Purified MAP from *Mytilus edulis* was obtained from Swedish Bioscience Laboratory (Floda, Sweden) and stored desiccated at -40°C. The amino acid composition according to the supplier is (per 1000 residues): 83 Asp, 74 Thr, 97 Ser, 64 Glu, 69 Pro, 132 Gly, 68 Ala, 50 Val, 25 Ile, 29 Leu, 30 Tyr, 12 Phe, 27 His, 115 Lys, 41 Arg, 41 Hyp and 70 3,4-dihydroxy-L-phenylalanine (DOPA). Acetic acid-urea polyacrylamide gel electrophoresis (PAGE) indicated that the preparation consisted of approximately 80% of the two DOPA containing proteins: MeFP-1 and MeFP-2 in equal quantities (Suci and Geesey, 1995). The rest of the mixture is comprised of lower molecular weight collagenic material. The protocol for the PAGE was performed using previously described methods and the identification of MeFP-1 (130kD) and MeFP-2 (45kD) were made according to previously published results (Waite and Benedict, 1984). Both MeFP-1 and MeFP-2 contain the unusual catecholic functionality DOPA.

Polystyrene (PS) (Aldrich Secondary Standard) was prepared as a 1.5%(W/V) solution in toluene. Poly(octadecyl methacrylate) (POMA) (Aldrich) was prepared as a 1.5%(V/V) solution in toluene. Bulk PS substrata was obtained from Plaskolite Inc. (Columbus, Ohio). All solvents, methanol, hexanes and toluene (Aldrich) were obtained as HPLC grade.

4.3 Preparation Of Substratum

4.3.1 Preparation Of Polymer Surfaces

PS and POMA polymer films were spin cast onto 1 cm x 1 cm PS fragments for all XPS and AFM studies. PS substrata were cleaned prior to spin casting by a solvent rinse, for 5 min each, in methanol and hexanes (Vargo, Thompson, Gerenser, Valentini, Aebischer, Hook and Gardella, 1992). Clean PS substrata were completely covered with

polymer solution and then immediately spun cast at 3500 rpm for 2 min. The polymer films were dried at room temperature for 24 h.

The underlying PS substrata contained trace constituents (<1% Zn, <2% Si, <2% O) besides PS that distinguished it from the pure, spun cast, PS obtained from Aldrich. XPS and time-of-flight secondary ion mass spectrometry of the polymer films, indicated that the films were continuous (i.e., no contaminants from the underlying polymer substratum were detected through the spin cast films).

4.3.2 Protein Adsorption Protocol

All protein films were deposited onto freshly prepared polymer surfaces after the 24 h drying period. A stock solution of 1 mg/ml MAP was prepared in dilute HCl (pH 2.9) with deionized, double-distilled water, deaerated with N₂. The stock solution was stored at 5°C. The polymer coated substrata were placed in glass cells with entrance and exit tubing ports to allow for protein adsorption and subsequent rinse without exposure to the air. All glassware used was cleaned with "piranha" solution, consisting of a 70:30 mix of concentrated H₂SO₄ and 30% H₂O₂ respectively. [WARNING: Piranha solution reacts violently, even explosively with organic materials (Dobbs, Bergman and Theopold, 1990).]

For the XPS studies, a 50 µl aliquot of the stock solution was delivered into the glass cell containing the substratum, followed immediately by a 0.95 ml aliquot of a pH 9.2 solution (Millipore water adjusted with NaOH). The final concentration of protein was 50 µg/ml at pH 8.5. After 1 h, the substratum was rinsed of any residual protein by flowing an aqueous solution at pH 8.5 (Millipore water adjusted with NaOH) through the reaction chamber at a rate of 100 ml/min for 1.5 min.

For the hydrated AFM studies, a much lower protein concentration was used to insure submonolayer coverage of the substratum, so that the spatial distribution of the

nucleation events could be resolved. For these experiments a 2 μl aliquot of the 1 mg/ml stock MAP solution was diluted in 0.998 ml of deionized double-distilled water at pH 2.9 to make a solution that was 2 $\mu\text{g/ml}$ MAP. A 25 μl aliquot of this solution was then delivered into the flow cell along with 0.975 ml of a dilute NaOH solution at pH 9.2, bringing the concentration of MAP to 25 ng/ml and the pH to pH 8.5. After 1 h, the substratum was rinsed of any residual protein by flowing an aqueous solution at pH 8.5 (Millipore water adjusted with NaOH) through the reaction chamber at a rate of 100 ml/min for 1.5 min.

4.4 Surface Characterization

4.4.1 Angle Resolved X-Ray Photoelectron Spectroscopy

Since protein adhesion to surfaces occurs in an aqueous environment, any reliable chemical analysis of the structure of the adsorbed proteins must be performed in the hydrated state. Therefore, cryostage sample handling techniques must be employed during the analysis of a hydrated surface in UHV. A hydrated surface can be frozen at liquid nitrogen (LN_2) temperatures and loaded onto a cold stage where the sample can be kept at LN_2 temperatures during analysis (Ratner, Weathersby, Hoffman, Kelly and Sharpen, 1978; Ratner and Castner, 1994). The structure of the adsorbed molecules at the surface are preserved in their hydrated state.

XPS spectra were obtained from a Physical Electronics instrument Model 5600 spectrometer (Physical Electronics, Eden Prairie, Minnesota). A 5 eV flood gun was used to offset charge accumulation on the samples. An 800 μm diameter spot size was scanned using a monochromatized Aluminum $\text{K}\alpha$ x-ray source at 350 Watts and a pass energy of 11.750 eV. For cold probe analysis, wet surfaces were removed from the glass cell after protein adsorption, rinsed, and mounted in the sample introduction chamber. The chamber was quickly purged with nitrogen and the sample was immediately brought into contact

with a liquid nitrogen cold finger before the sample could dehydrate. The sample remained in contact with the cold finger for 30 min followed by immediate sample transfer to the cold stage in the analytical chamber. The cold stage was maintained at -120°C during analysis. Samples that were dehydrated at room temperature were removed from the final rinse and dried under an atmosphere of pure, dry N_2 in the sample introduction chamber of the XPS. Before the variable angle study was conducted an initial 80° (near normal) high resolution spectra was collected. Depth profiles were performed using variable angle XPS data collected at take-off angles of 15° , 22° , 35° and 80° from the surface. The elemental compositions at the initial 80° survey were compared with the final 80° angle study to ensure no x-ray damage of the surface had occurred during analysis. Elemental quantification was calculated on peak areas from the C1s, N1s, and O1s core levels. Relative peak areas were calculated by fitting the high resolution C1s, N1s and O1s peaks with Gaussian functions. The binding energy scale was referenced by setting the CHx peak maximum in the C1s spectrum to 285.0 eV (Ratner and Castner, 1994).

The relative atomic concentrations at the different take-off angles were used with the calculated escape depths of the photoelectrons for each angle to gain insight on the elemental depth distribution of the proteins adsorbed to the polymer surfaces. The escape depths were calculated using parameters for organic compounds to calculate the inelastic mean free paths and subsequently the escape depths using equations previously defined (Seah and Dench, 1980).

4.4.2 Atomic Force Microscopy Imaging

In order to image hydrated biological molecules on a surface using AFM, a mode of operation called fluid Tapping ModeTM was used. In fluid Tapping ModeTM the AFM cantilever operates at a known frequency (Zhong, Inniss, Kjoller and Elings, 1993). As the probe tip approaches the surface its oscillations are dampened. Since the tip only

intermittently "taps" the surface, this mode of operation is characterized by overall weak tip-sample interactions. This allows minimal disturbance of the adsorbed molecules being imaged.

All surfaces were imaged using a BioscopeTM AFM (Digital Instruments, Inc. Santa Barbara, CA). The instrument was used in fluid Tapping modeTM using thin legged, triangular, microfabricated silicon nitride cantilevers which were 100 μm in length, with a nominal spring constant of 0.38 N/m and possessing integrated pyramidal tips. The samples were attached to the fluid cell using a cyanoacrylate adhesive and dried in a laminar flow hood. The images were recorded as 1 μm x 1 μm and 2 μm x 2 μm scan areas with 512 x 512 data points per scan area. Images were recorded at scan rates of 0.8 to 6.1 $\mu\text{m/s}$. Scan rates were optimized to minimize hysteresis between the forward and return traces of the probe. All images were stable with time and reproducible. For analysis, images were low-pass filtered, flattened to remove sample tilt, and plane fit to remove sample bow arising from hysteresis in the piezocrystal.

4.5 Results

4.5.1 Atomic Force Microscopy Imaging

MAP adsorbed to clean PS and POMA from solutions with bulk protein concentrations of 25 ng/ml were imaged in fluid Tapping ModeTM using AFM. Figures 15a and 15b show AFM contour images of 1 μm x 1 μm areas of the PS and POMA substrata before MAP adsorption. Before MAP adsorption, the PS and POMA surfaces are extremely smooth with few surface features and root-mean-square (RMS) surface roughness values of 0.52 ± 0.13 nm and 1.06 ± 0.08 nm, respectively. Figure 16 shows AFM contour images of 1 μm x 1 μm areas of PS and POMA substrata after MAP adsorption. Adsorption of MAP to the PS surface resulted in the formation of closely packed, repeating structures as is shown in Figure 16a. Cross sectional analysis of these

features revealed a near monolayer structure, making it difficult to differentiate between adjoining protein structures. The heights of these features are less than 1 nm. MAP adsorbed to the POMA surface displays very different protein features that appear to be linearly ordered as revealed in the $1\ \mu\text{m} \times 1\ \mu\text{m}$ scan area in Figure 16b. An additional scan performed at a right angle to the first showed these features to rotate 90° , indicating that the linear features observed were not an artifact of the AFM probe tip rastering across the surface. Cross-sectional analysis reveals that these features have an average height of 5.0 ± 0.7 nm with lateral dimensions of 67 ± 17 nm (minor axis) and 177 ± 26 nm (major axis).

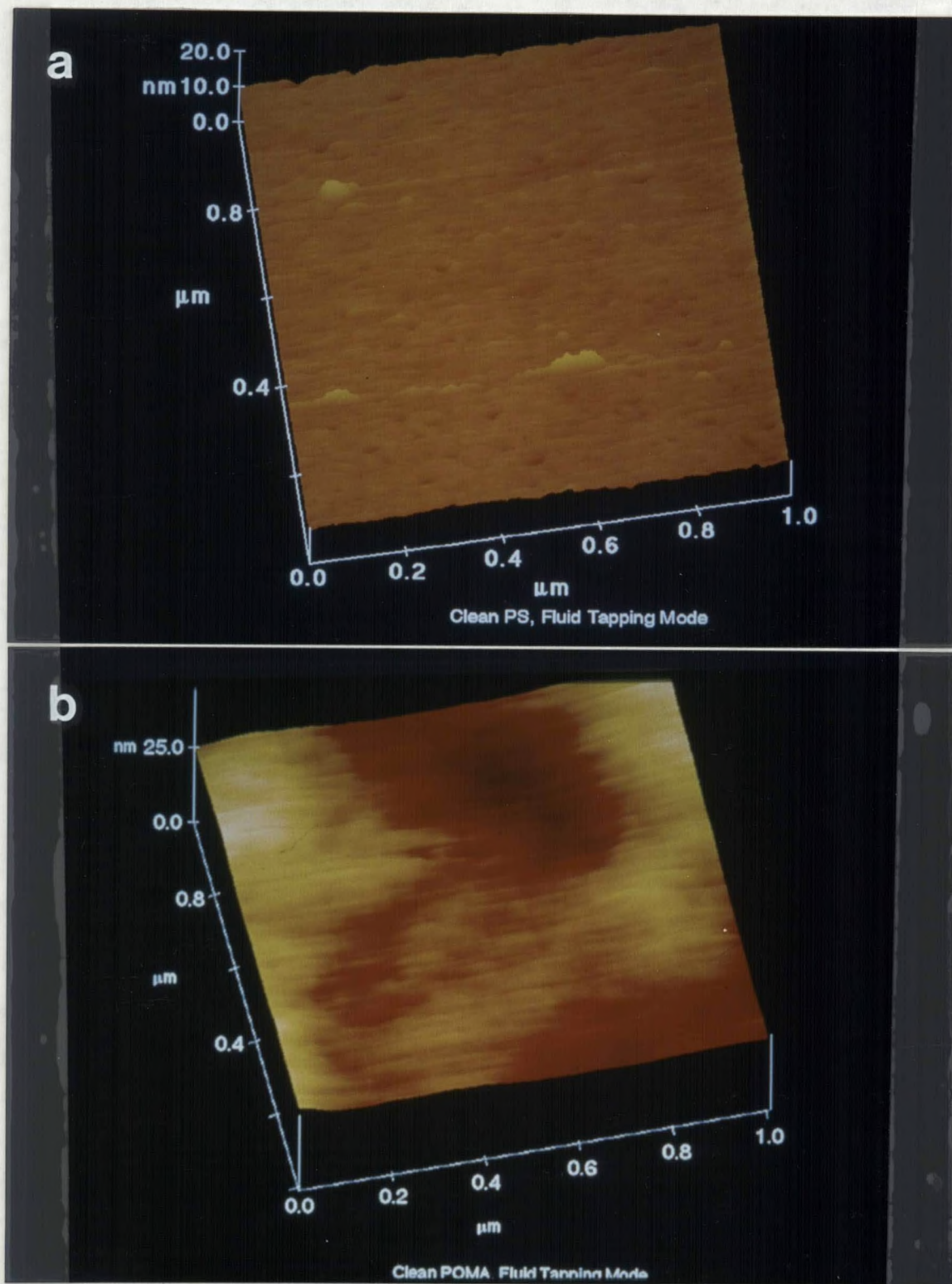


Figure 15. Fluid Tapping Mode™ AFM contour images of; 1 μm x 1 μm area of PS (a) and POMA (b) before protein adsorption.

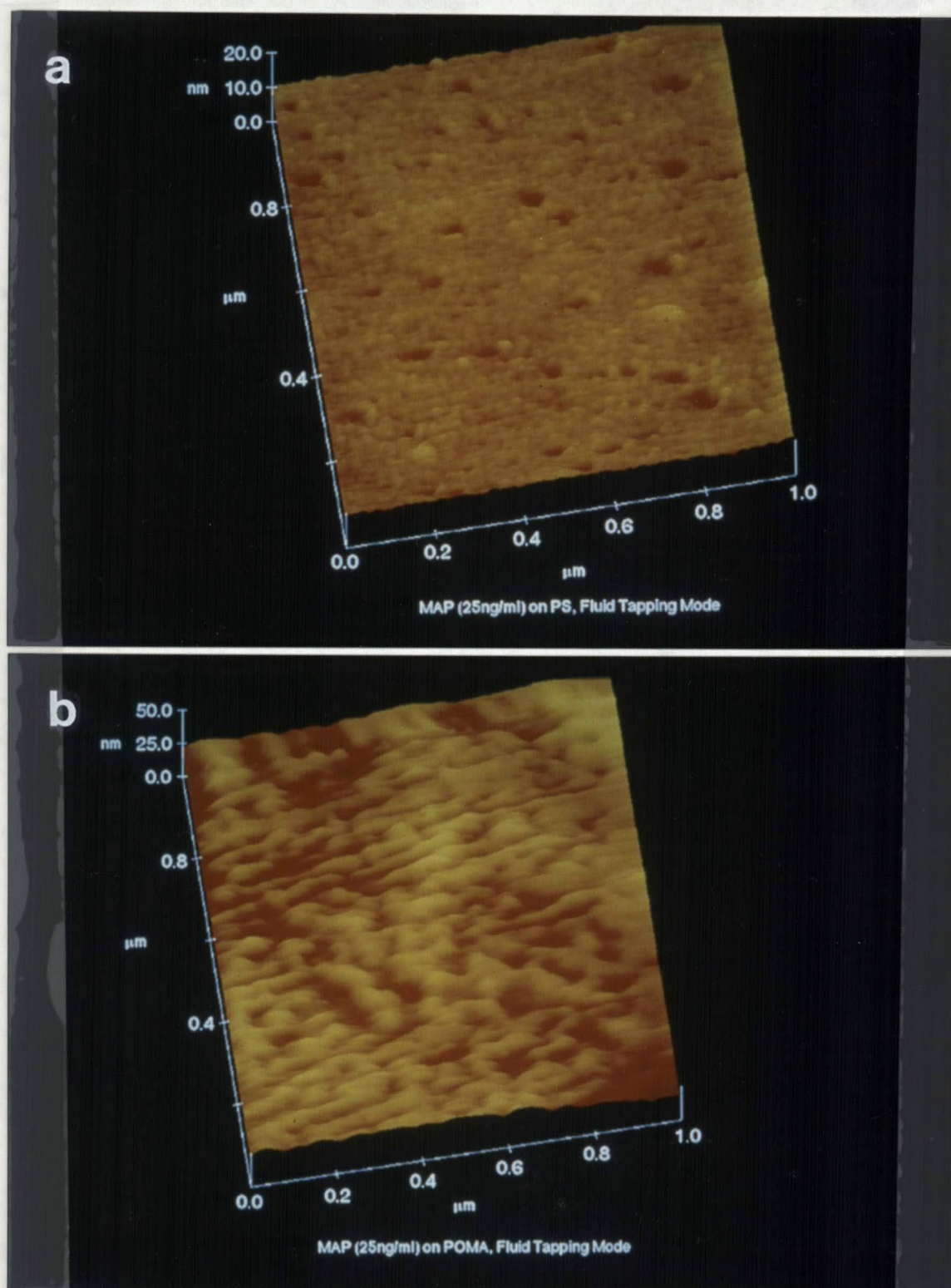


Figure 16. Fluid Tapping ModeTM AFM contour images of; $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$ area of MAP adsorbed to PS (a) and POMA (b).

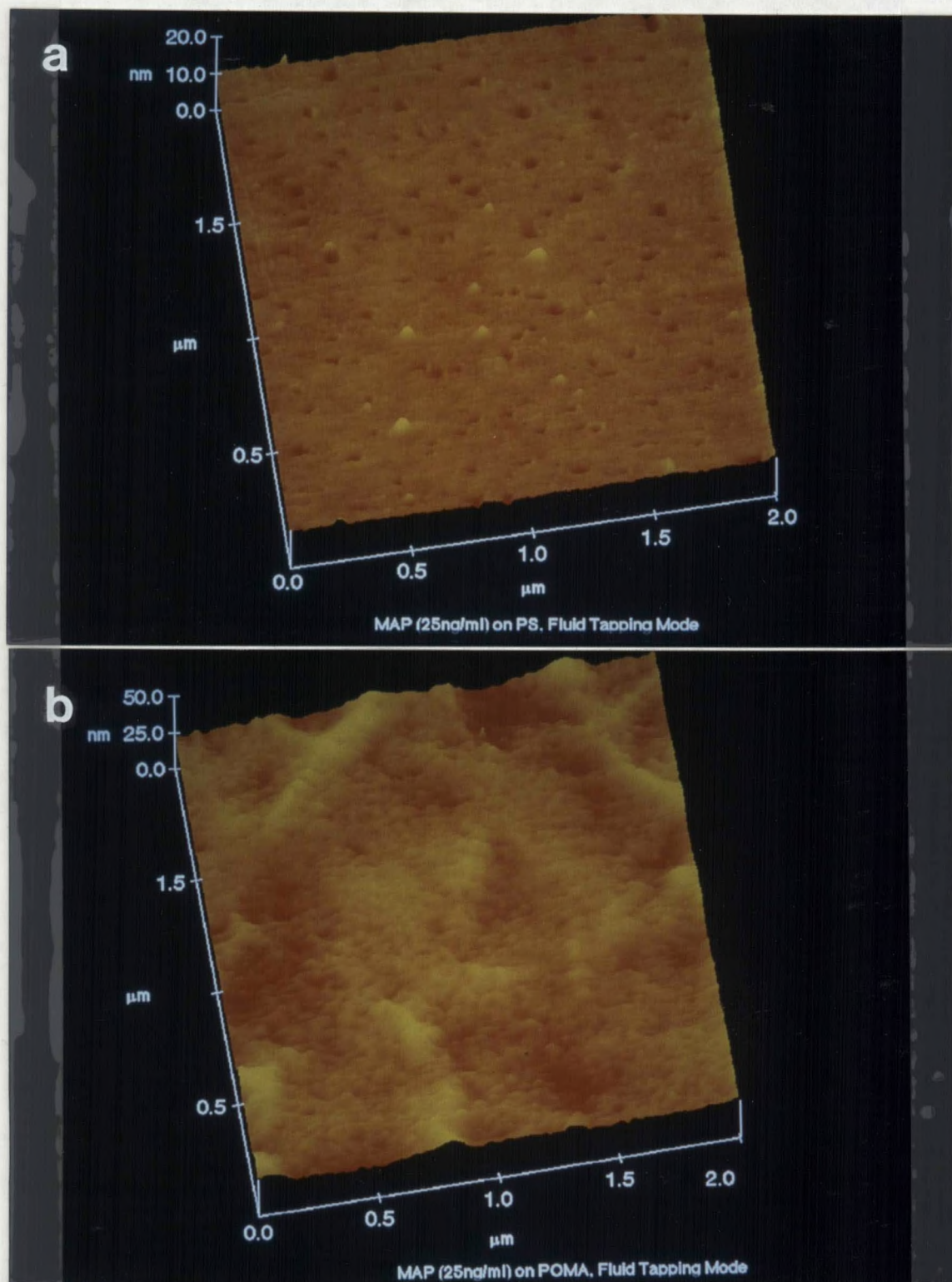


Figure 17. Fluid Tapping ModeTM AFM contour images of; 2 $\mu\text{m} \times 2 \mu\text{m}$ area of MAP adsorbed to PS (a) and POMA (b).

The $2\text{ }\mu\text{m} \times 2\text{ }\mu\text{m}$ scan area of MAP adsorbed to PS, shown in Figure 17a, reveals that the features observed at high magnification cover larger areas of the surface, suggesting that the surfaces are homogeneous and have continuous protein coverage. The $2\text{ }\mu\text{m} \times 2\text{ }\mu\text{m}$ scan area of MAP adsorbed to POMA, shown in Figure 17b, shows the protein features on POMA are also very homogeneous. However, there appears to be larger protein domains covering the surface, which might suggest extensive cross-linking of the adsorbed protein. These features extend $6.2 \pm 1.7\text{ nm}$ in height with lateral dimensions of $173 \pm 35\text{ nm}$ (minor axis), with some as long as 760 nm .

4.5.2 Angle Resolved X-Ray Photoelectron Spectroscopy

Angle dependent XPS, performed at room temperature and LN_2 temperatures, of the surfaces studied in Figures 15, 16 and 17 reveals further differences in MAP adsorption to PS and POMA surfaces. A detailed examination of the C1s region of clean PS and POMA at a 30° take-off angle and at LN_2 temperatures is shown in Figure 18. MAP adsorbed to PS at take-off angles of 80° and 22° at LN_2 temperatures and at 22° at room temperature are given in Figure 19. MAP adsorbed to POMA at a take off angle of 80° and 22° at LN_2 temperatures and at 22° at room temperature are given in Figure 20.

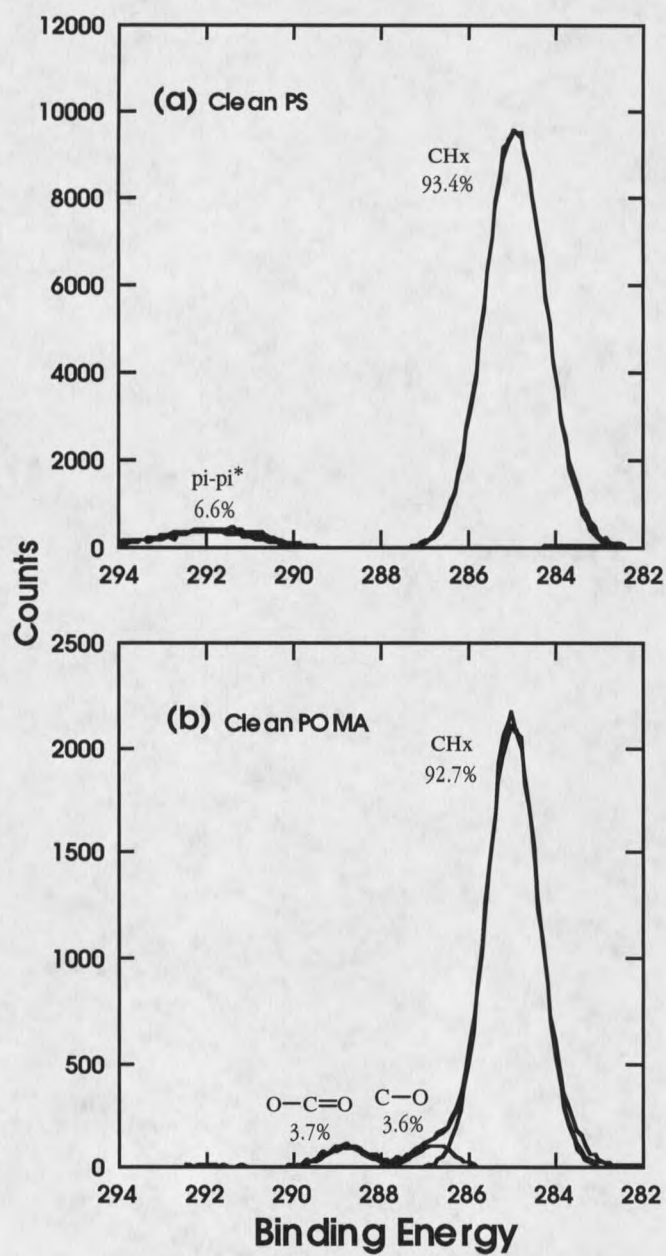


Figure 18. C1s XPS spectra of clean PS (a) and POMA (b) at take-off angles of 30° at LN₂ temperature.

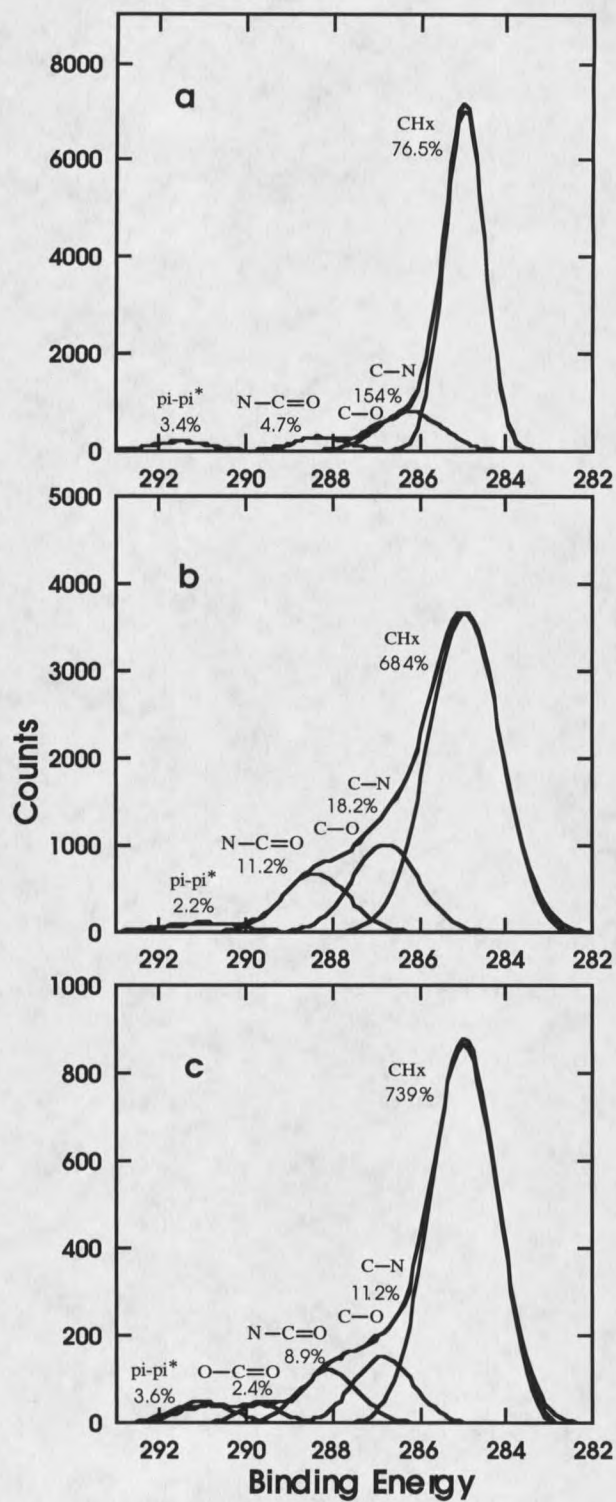


Figure 19. C1s XPS spectra of MAP adsorbed to PS at 80° (a) and 22° (b) take-off angles at LN₂ temperature and 22° (c) at room temperature.

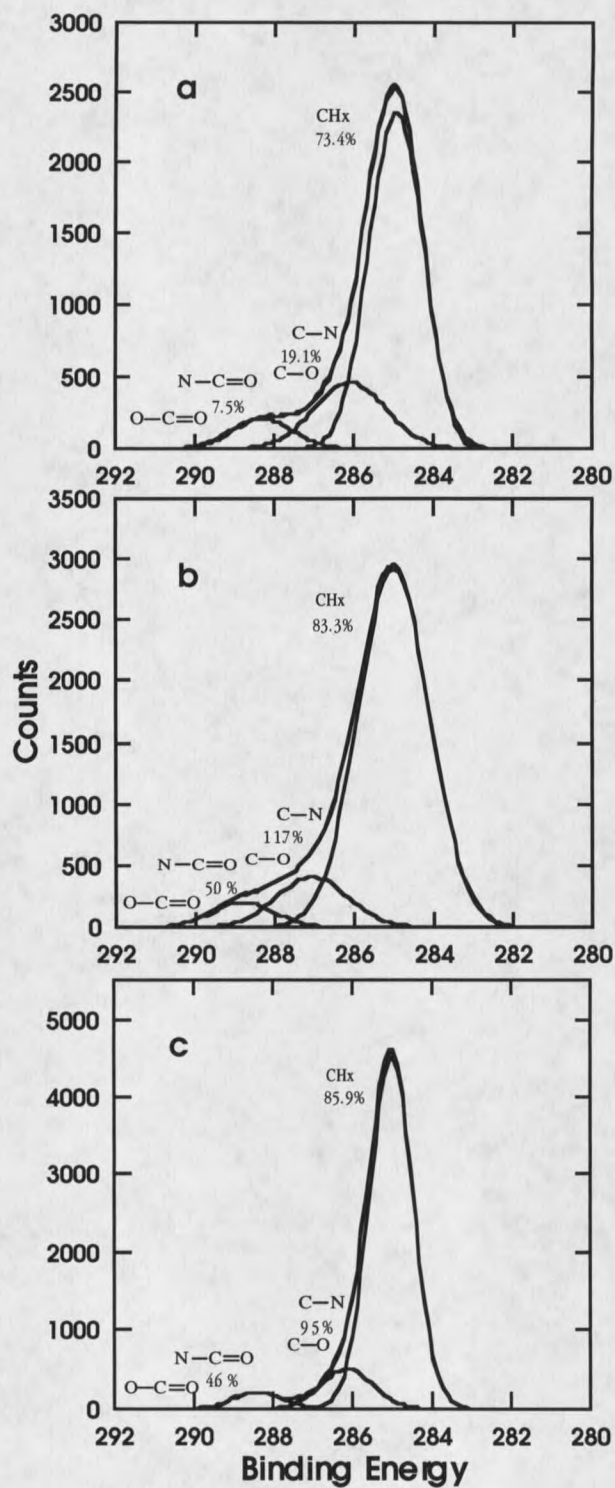


Figure 20. C1s XPS spectra of MAP adsorbed to POMA at 80° (a) and 22° (b) take-off angles at LN₂ temperature and 22° (c) at room temperature.

Each peak is contributed to by different chemical groups. The C1s peak at a binding energy of 291.0-291.6 eV is attributed to the π - π^* transition in the aromatic rings of the polystyrene. The peak at 288.0-288.8 eV arises from the N-C=O and O-C=O functionalities on the protein and the methacrylate chain, respectively. The C1s peak at a binding energy of 286.2-287.0 eV is attributed to the C-N and C-O functionalities in the protein and the C-O-C functionality of the POMA. The dominant peak in both sets of spectra is at 285.0 eV. This peak originates from the aliphatic carbon in both the polymers and the protein.

The C1s region of MAP adsorbed to PS, at LN₂ temperature and at a take-off angle of 80° (Figure 19a) reveals a decrease in the aliphatic peak at 285.0 eV and an increase in the 286.2 eV and the 288.2 eV peaks when compared to clean PS (Figure 18a). This indicates MAP contribution to the C1s region. At a take-off angle of 22° at LN₂ temperature, (Figure 19b) there is a shift in the peak at 286.2 eV towards 286.8 when compared to the spectrum collected at 80°. This peak shift suggests an increase in the signal from the C-O functionalities that are present on the protein. There is also a further increase in the 286.8 eV and 288.2 eV peaks, as compared to the spectrum at 80°, indicating that at the angle approaching glancing the signal from the PS is decreasing while that of the adsorbed protein is increasing. The C1s region of MAP adsorbed to PS and dehydrated at room temperature (Figure 19c) shows an increase in the 291.6 eV peak that is attributed to the π - π^* transition of the aromatic rings of the PS. There is also a decrease in the peak area of the 286.8 eV and the 288.1 eV peaks from the adsorbed protein. This indicates that more of the PS is contributing to the spectra when the surface is dehydrated at room temperature as compared to LN₂ temperature. Furthermore, a peak at 289.0 eV (acid or ester functionality) appears in the spectra upon dehydration that is not part of the MAP or PS C1s spectra, indicating other adsorbed species have migrated to the surface upon dehydration.

The C1s region of MAP adsorbed to POMA, at LN₂ temperature and at a take-off angle of 80° (Figure 20a), reveals a shift in the peaks at 286.7 eV and 288.8 eV (clean POMA) towards 286.1 eV and 288.3 eV respectively. This is a result of an increase in the signal from the C-N functionalities that are present from the adsorbed protein. There is also a decrease in the aliphatic peak at 285.0 eV and an increase in the 286.1 eV and 288.3 eV peaks when compared to clean POMA (Figure 18b). This also indicates MAP contribution to the C1s region. At a take-off angle of 22° at LN₂ temperature, (Figure 20b) there is a shift in the 286.1 eV and 288.3 eV peaks back to 286.7 eV and 288.8 eV respectively. This could be a result of an increase in the signal from the C-O functionalities that are present from the adsorbed protein. However there is no significant increase in the peak areas attributed to by the protein. The fact that the AFM images show homogeneous coverage and there is no significant difference in these peak areas at the two different take-off angles suggest that the adsorbed protein layer on the POMA is much less densely packed than on the PS surface, where the signal intensity at the 22° take-off angle (LN₂) looks like mostly MAP contribution. The C1s region of MAP adsorbed to POMA and dehydrated at room temperature (Figure 20c) shows a decrease in the 286.5 eV and 288.5 eV peak and almost looks like POMA. This indicates that more of the POMA is contributing to the spectra when the surface is dehydrated at room temperature than when it is at LN₂ temperature during analysis. It was observed that at LN₂ temperature during XPS analysis, the high resolution C1s peaks were broader than the same spectra taken at room temperature. This might be attributed to less efficient charge compensation at LN₂ temperatures at the lower take-off angles. However, how this would affect signal intensity is currently unknown.

The relative atomic concentrations at the different take-off angles were used with the calculated escape depths of the photoelectrons for each angle to gain insight on the elemental depth distribution of the proteins adsorbed to the polymer surfaces. The escape

depths were calculated using parameters for organic compounds to calculate the inelastic mean free path and subsequently the escape depth using equations previously defined (Seah *et al.*, 1980). Table 4 and 5 show the atomic concentration of MAP adsorbed to PS and POMA at room temperature and LN₂ temperature, respectively.

Table 4: Atomic Concentration of MAP Adsorbed to PS and POMA, dehydrated at Room Temperature.

<u>Atomic Percent</u>					
<u>Sample</u>	<u>Take-off Angle</u>	<u>Sampling Depth</u>	<u>C</u>	<u>O</u>	<u>N</u>
MAP	80	84.3	80.07	13.88	6.05
Adsorbed to	35	49.1	78.67	14.02	7.31
Polystyrene	22	32.1	74.92	16.90	8.18
	15	22.2	74.14	17.57	8.29
MAP	80	84.3	88.66	10.02	1.32
Adsorbed to	35	49.1	88.10	10.87	1.03
POMA	22	32.1	90.25	8.54	1.21
	15	22.2	91.30	7.60	1.09

Table 5: Atomic Concentration of MAP Adsorbed to PS and POMA, dehydrated at LN₂ Temperature.

<u>Atomic Percent</u>					
<u>Sample</u>	<u>Take-off Angle</u>	<u>Sampling Depth</u>	<u>C</u>	<u>O</u>	<u>N</u>
MAP	80	84.3	79.21	14.46	6.33
Adsorbed to	35	49.1	74.78	16.15	9.08
Polystyrene	22	32.1	68.73	19.41	11.85
	10	22.2	66.95	20.01	13.04
MAP	80	84.3	79.10	16.55	4.34
Adsorbed to	35	49.1	79.76	15.61	4.63
POMA	22	32.1	77.69	17.00	5.31
	15	22.2	77.41	16.68	5.92

Figure 21 shows a plot of the atomic concentration of MAP adsorbed to PS (Figure 21a) and POMA (Figure 21b) at take-off angles of 80° ($84.3 \text{ \AA}$), 35° ($49.1 \text{ \AA}$), 22° ($32.1 \text{ \AA}$) and 15° ($22.2 \text{ \AA}$).

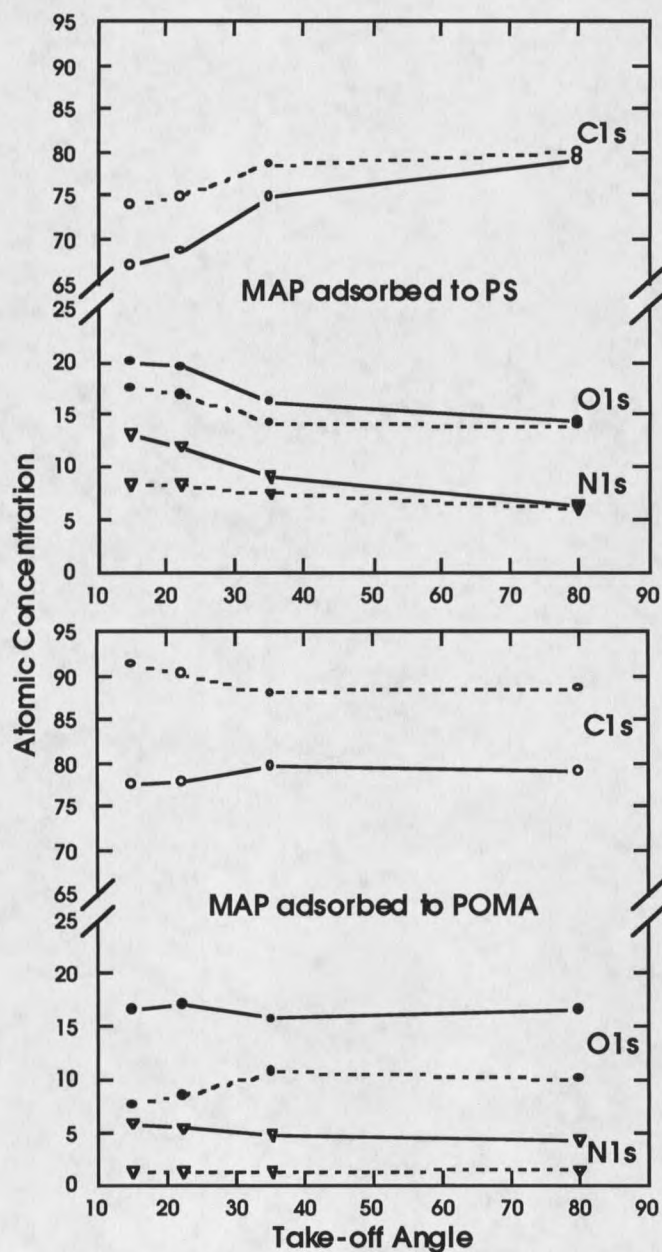


Figure 21. Summary of angle resolved XPS data of MAP adsorbed to PS (top) and POMA (bottom) at LN₂ temperature (solid line) and room temperature (broken line).

Both the atomic concentration for the LN₂ temperature analysis and the dehydrated room temperature analysis are shown. At all depths, on both PS and POMA exposed to MAP, the atomic concentration of nitrogen and oxygen was lower when the samples were dehydrated and analyzed at room temperature as compared to the surfaces analyzed at LN₂ temperatures. This also suggests that more of the substratum is contributing to the spectra upon dehydration of the adsorbed MAP, which decreases the signal intensity of the adsorbed MAP. When MAP adsorbed to PS was analyzed at LN₂ temperature and room temperature, the surface dehydrated at room temperature experienced an average 2.6% loss in nitrogen and a 1.9% loss in oxygen upon dehydration. Furthermore, the atomic concentrations for the LN₂ and room temperature studies converge at the 80° take-off angle for MAP adsorbed to PS. In contrast, when MAP adsorbed to POMA was analyzed at LN₂ temperatures and at room temperature, the surface experienced a much greater loss of nitrogen and oxygen upon dehydration of 3.9% and 7.2% respectively. Furthermore, the atomic concentrations for the LN₂ and room temperature studies do not converge at the 80° take-off angle for MAP adsorbed to POMA.

4.6 Discussion

The results presented here demonstrate that the functional groups present at a polymer surface can influence protein-protein and protein-surface interactions. The data also indicates that dehydration of the adsorbed proteins on the polymer surface can give rise to differences that are detected by XPS and AFM. The differences in substratum surface chemistry and the differences that arise from dehydration of the adsorbed proteins are a reflection of the behavior of the adsorbed proteins and their interactions with the substratum.

Interactions that drive protein adsorption can be classified according to whether the contribution towards energy minimization is primarily enthalpic or entropic. For the

purposes of this discussion these interactions will be referred to as specific and non-specific interactions, respectively (not to be confused with receptor ligand-interactions). Specific interactions involve the interaction of functionalities of the surface with the protein which involve a large change in enthalpy, such as; covalent, ionic and hydrogen bonding as well as charge-transfer (donor-acceptor) interactions. Non-specific interactions involve a binding mechanism that leads to an energy minimization of the system which is primarily due to an entropy maximization, such as hydrophobic interactions or very weak, non-specific enthalpic interactions such as van der Waals forces. There are several specific interactions that have been proposed to play important roles in MAP-MAP and MAP-surface interactions, including: hydrogen bonding, metal-ligand complexes, the formation of Michael-type addition compounds derived from o-quinones, and charge transfer complexes (Waite, 1987). The interactions that are available in the polymer-protein system studied here are limited. The PS surface provides a hydrophobic surface with an aromatic character and a medium surface free energy; the POMA surface provides a hydrophobic low energy surface with an aliphatic functionality.

Specific protein-surface interactions can be encouraged by careful surface design. Non-specific interactions are much more difficult to control since they involve multiple types of interactions between the solvent, the surface and other adsorbed species which result in an energy minimization upon adsorption. For example, proteins can orient their hydrophobic residues towards a hydrophobic surface, displacing ordered water. This minimizes the interfacial free energy of the system and provides the driving force for the protein adsorption. This also leaves the hydrophilic residues of the protein available for protein-protein or protein-solvent interactions which may further stabilize the adsorbed proteins at the surface. An increase in entropy due to displacement of ordered water near the interface may also be a factor in adsorption. These types of protein-surface interactions

result in a tenaciously bound protein layer with no specific interactions between the surface and the adsorbed protein layer.

Adsorption of a series of aromatic compounds has been previously studied and it was found that the orientation of the adsorbed aromatic domains followed several rules (Waite, 1987). In the absence of chemisorbable functional groups that interfere with the aromatic framework, and in the absence of bulky electronegative constituents on the aromatic framework, the o-quinones were found to adsorb parallel to the surface. For human blood plasma albumin and bovine pancreas ribonuclease adsorption to PS, it has been shown that in the absence of dominant enthalpic interactions between specific groups of the surface and the protein, the proteins affect the overall adsorption by dehydrating or displacing water from the surface (Norde and Lyklema, 1979). Such adsorption increases the entropy of the system providing the driving force for adsorption. Since there is no dominant enthalpic interaction between MAP and the POMA surface (i.e., there exists no functionalities that would provide for hydrogen bonding, ionic, or donor-acceptor type interactions with the surface) the observed adsorption of MAP to POMA is likely to be driven primarily by non-specific interactions.

There are two specific interactions that could be responsible for the protein-protein interactions in MAP: hydrogen bonding and a charge transfer interaction between the aromatic residues of MAP. It has been previously found that o-diphenols (like DOPA) can irreversibly displace ordered water from surfaces (Soriaga and Hubbard, 1982). In the case of MAP adsorption, water provides the driving force for the adsorption to both PS and POMA as described above. Then at an elevated pH of 8.5 (the pH during adsorption), the catechol functionality on the DOPA can undergo a spontaneous reverse dismutation to the o-quinone that is capable of interacting through a quinhydrone charge-transfer complex. Since the aromatic residues in MAP are not enthalpically involved in adsorption on the POMA surface, these residues are free to interact with themselves, driving a cross-linking

reaction. Evidence for this behavior can be seen in the AFM images, as is shown schematically in Figure 22. Adsorbed MAP on POMA displays a highly crosslinked surface that appears to be almost web like. In contrast, the MAP adsorbed to the PS appears to consist of individual protein molecules. In contrast to the POMA surface, the PS surface has considerable aromatic character. Therefore, after MAP adsorbs to the surface, the protein molecules may interact specifically with PS through pi-pi overlap interactions further strengthening the interaction, as is shown in Figure 22. This is reflected in the AFM images of MAP on PS which show little evidence of protein aggregation but rather individual, tightly packed structures.

We have previously reported an XPS and AFM investigation of MAP adsorbed to PS and POMA in the dehydrated state (Baty, Suci, Tyler and Geesey, 1995). Similar surface features were observed here but there was an apparent loss in structure of the adsorbed protein after dehydration. This can be seen as an increase in the topography, indicating the adsorbed protein layer may have collapsed upon dehydration. If these dehydrated images are compared with the hydrated images reported here, MAP adsorption to the PS surface retains its lateral spatial distribution across the surface but loses vertical structure upon dehydration. MAP adsorbed to POMA not only loses vertical structure but also loses spatial distribution across the surface when dehydrated, exposing the underlying substratum. This supports the hypothesis that the MAP adsorbed to the PS surface has a very specific means of interaction, whereas MAP adsorption to POMA is non-specific, and requires the driving force that is provided by the presence of water.

Dehydration would also explain the differences observed between the room temperature and LN₂ temperature XPS data. A protein that is adsorbed to a surface through non-specific types of interactions (as defined above) might be expected to exhibit a drastic change in the adsorbed film structure upon dehydration, since the entropic

contribution towards energy minimization has been partially removed, as is illustrated in Figure 22 for MAP adsorbed to POMA.

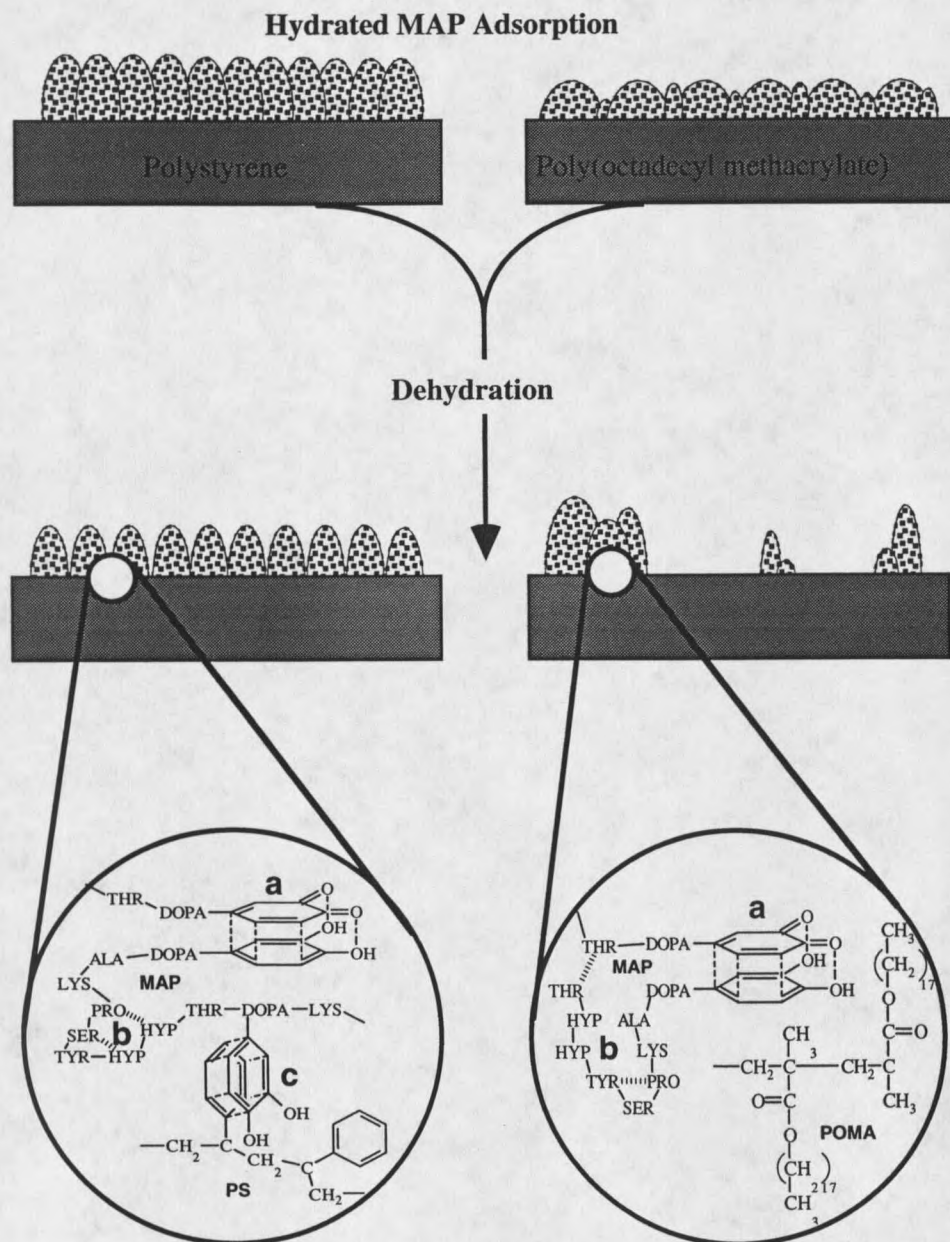


Figure 22. Schematic representation of the AFM images observed as related to the specific and non-specific mechanisms available for adsorption; a = quinhydrone charge transfer complex, b = hydrogen bonding, c = pi-pi overlap interactions.

The deficiency in atomic concentration of the protein (nitrogen and oxygen) after dehydration, as was shown in Figure 21, is probably due to a loss of structure with depth on both surfaces. The greater decrease in atomic concentration on the POMA surface may also reflect the adsorbed proteins loss of lateral spatial distribution, further increasing the signal from the POMA and decreasing the signal from the MAP. The stability of the adsorbed proteins on the PS surface is reflected in the densely packed structures seen in the AFM, hydrated and dehydrated. This may also explain the converging atomic concentrations for the two studies displayed in Figure 21. The converging atomic concentrations indicate that at the 80° take-off angle we are sampling the same amount of protein on the surface whether they are at room or LN₂ temperature. The changes seen in the adsorbed architecture on the PS surface can only be detected, with XPS, at the shallower sampling depth where the signal intensity arises mainly from the protein. The instability of the adsorbed proteins on the POMA surface is reflected in the loss of lateral spatial distribution upon dehydration, seen in the AFM. The difference between the room and LN₂ temperature adsorbed architecture is great enough to be detected at the 80° take-off angle and becomes even greater as the sampling depth decreases.

The LN₂ temperature XPS analysis and hydrated AFM images demonstrate that differences in substratum chemistry will influence protein adsorption. This research shows that when MAP is subjected to dehydration on a surface, changes in the adsorbed architecture affect the XPS and AFM data. However, the magnitude of the change depends on what types of interactions are present between the protein and the surface. It would be expected, then, that irreversible protein adsorption involving specific, enthalpically driven interactions such as covalent, ionic, hydrogen bonding or donor acceptor type interactions will experience much less disruption upon dehydration than non-specific, entropically driven interactions that require the presence of water as the driving force for adsorption. It should also be noted that at the lower take-off angles at LN₂ temperature charge

compensation on an insulating surface appears to be affected. How much charge compensation and subsequently the signal intensity is affected by the decrease in temperature and/or the presence of frozen water is uncertain and remains a question to be investigated. Such differences can be correlated through these complementary analytical techniques without introducing artifacts resulting from desiccation of the adsorbed proteins.

CHAPTER 5

SUMMARY

The adsorption of Mussel Adhesive Protein (MAP) from the marine mussel *Mytilus edulis* has been investigated on polystyrene (PS) and poly(octadecyl methacrylate) (POMA) surfaces using angle dependent x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). AFM images acquired in the dehydrated state using contact mode are compared with images acquired in the hydrated state using fluid Tapping Mode™ to assess the contribution that hydration has on the adsorbed proteins. To further characterize the adsorbed protein layer, XPS analysis was performed at liquid nitrogen (LN₂) temperature without dehydrating the samples and at room temperature after the surfaces were dehydrated. The adsorption pattern of MAP in the dehydrated state, revealed by AFM, is similar to that of the hydrated images. However there is an apparent loss of structure and change in the spatial distribution of the adsorbed proteins. MAP adsorbed to PS showed a collapse of the adsorbed proteins on the surface upon dehydration, but no loss in lateral spatial distribution. In contrast, MAP adsorbed to POMA showed a loss of lateral spatial distribution upon dehydration. Angle resolved XPS shows differences in nitrogen composition with depth for MAP adsorbed to PS and POMA at liquid nitrogen temperature. Angle resolved XPS at room temperature shows a decrease in Nitrogen composition over the LN₂ temperature studies indicating that hydration plays an important role in stabilizing the adsorbed proteins on both the PS and POMA surfaces. The differences observed upon dehydration can be attributed to the strength of the interactions between the MAP and the two surfaces. The AFM and XPS data indicate that the adsorbed MAP is stabilized on the surface of the PS through specific interactions preventing the protein from losing lateral spatial distribution across the surface. The adsorbed MAP on the POMA surface is representative of a loosely bound protein layer that is adsorbed through non-specific types

of interactions allowing the protein to lose much of its lateral spatial distribution when dehydrated. This data demonstrates that the surface chemistry of the polymer films can influence protein-protein and protein-surface interactions. These techniques will allow studies on microbial adsorption that can now be correlated to differences in the protein-adsorption. Through the use of surface analysis techniques such as the cold probe techniques in XPS and the fluid Tapping ModeTM in AFM a greater understanding can be gained on the interactions governing protein adsorption to submerged surfaces and the subsequent molecular interactions that govern microbial adhesion.

REFERENCES

- Andrade, J.D. 1985. Principles of protein adsorption. pp. 1-80. In Andrade, J.D., Ed., *Surface and Interfacial Aspects of Biomedical Polymers*, vol. 2. Plenum Press, New York.
- Atkins, P.W. 1990. Quantum theory. pp. 287. In Atkins, P.W., Ed., *Physical Chemistry*, 4th edition. W.H. Freeman and Company, New York.
- Baier, R.E. 1980. Substrata influences on the adhesion of microorganisms and their resultant new surface properties. pp. 59-104. In Bitton, G. and Marshall, K.C., Eds., *Adsorption of microorganisms to surfaces*. John Wiley and Sons, Inc., New York.
- Benedict, C.V. and Waite, J.H. 1986. Location and analysis of byssal structural proteins of *Mytilus edulis*. *Journal of Morphology*. **189**: 171-181.
- Binnig G.B., Quate, C.F. and Gerber, C.H. 1986. Atomic force microscope. *Physical Review Letters*. **56**: 930-936.
- Briggs, D. 1977. C1s Binding energy shifts. pp. 277. In Briggs, D. Ed., *Handbook of X-ray and Ultraviolet Photoelectron Spectroscopy*. Heydon Press, London.
- Fadley, C.S., 1981. Basic concepts of x-ray photoelectron spectroscopy. pp. 23-74. In Brundle, C.R. and Baker, A.D., Eds., *Electron Spectroscopy: Theory, Techniques and Applications*, Vol. 2. Academic Press, London.
- Carlson, T.A. 1975. Photoelectron spectroscopy of the outer (and inner) shells. pp. 99-217. In Carlson, T.A., Ed., *Photoelectron and Auger Spectroscopy*. Plenum Press, London.
- Characklis, W.G. 1990. Microbial fouling. pp. 524-584. In Characklis, W.G. and Marshall, K.C., Eds., *Biofilms*. John Wiley and Sons, New York.
- Christie, A.B. 1990. X-ray photoelectron spectroscopy. pp. 128-137. In Waals, J.M., Ed., *Methods of Surface Analysis*. University Press, Great Britain.
- Corpe, W.A. 1977. Biofouling and corrosion symposium. In *Proceedings of the Ocean Thermal Energy Conversion (OTEC)*, Washington D.C..
- Dobbs, D.A., Bergman, R.G. and Theopold, K.H. 1990. Piranha solution explosion ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$). *Chemical Engineering News*. **68**(17): 2.
- Dousseau, F., Therien, M. and Pezolet, M. 1989. On the spectral subtraction of water from the FT-IR: spectra of aqueous solutions of proteins. *Applied Spectroscopy*. **43**: 538-542.
- Drake, B., Prater, C.B., Weisenhorn, A.L., Gould, S.A.C., Albrecht, T.R., Quate, C.F., Cannell, D.S., Hansma, H.G. and Hansma, P.K. 1989. Imaging crystals,

- polymers and processes in water with the atomic force microscope. *Science*. **243**: 1586-1589.
- Fink, D.J., Hutson, T.B., Chittur, K.K. and Gendreau, R.M. 1987. Quantitative surface studies of protein adsorption by infrared spectroscopy. *Analytical Biochemistry*. **165**: 147-154.
- Fulkerson, J.P., Norton, L.A., Gronowicz, G, Picciano, P., Massicotte, J.M. and Nissen, C.W. 1990. Attachment of epiphyseal cartilage cells and 17/28 rat osteoblasts using mussel adhesive protein. *Journal of Orthopedic Research*. **6**: 793-798.
- Grande, D.A. and Pitman, M.I. 1988. The use of adhesives in chondrocyte transplantation surgery: preliminary studies. *Bulletin of the Hospital for Joint Diseases Orthopedic Institute*. **2**: 140-148.
- Hansen, D.C., Luther III, G.W. and Waite, J.H. 1994. The adsorption of the adhesive protein of the blue mussel, *Mytilus Edulis L*, onto type 304L stainless steel. *Journal of Colloid and Interface Science*. **168**: 206-216.
- Healy, K.E., Thomas, C.H., Rezania, A., Kim, J.E., McKeown, P.J., Lom, B. and Hockberger, P.E. 1995. Kinetics of primary bone cell organization and mineralization on materials with patterned surface chemistry. *Biomaterials: Special Issue on Tissue Engineering*.
- Jakobsen, R.J. and Wasacz, F.M. 1987. Effects of the environment on the structure of adsorbed proteins: Fourier transform infrared spectroscopic studies. pp. 339-361. In Brash J.L. and Horbett, T.A., Eds., *Proteins at Interfaces: Physiochemical and Biochemical Studies*. American Chemical Society, Washington, D.C..
- Kaiden, K., Matsui, T. and Tanaka, S. 1987. A study of the amide III band by FT-IR spectrometry of the secondary structure of Albumin, Myoglobin, and γ -Globulin. *Applied Spectroscopy*. **41**: 180-192.
- Lehninger, A.L., Nelson, D.L. and Cox, M.M. 1993. Water: Its effect on dissolved biomolecules. In Lehninger, A.L., Nelson, D.L. and Cox, M.M., Eds., *Principles of Biochemistry*. pp. 88-90. Worth Publishers, New York.
- Little, B.J. 1985. Factors influencing the adsorption of dissolved organic material from natural waters. *Journal of Colloid and Interface Science*. **108**(2): 331-339.
- Marshall, K.C. 1992. Biofilms: An overview of bacterial adhesion, activity, and control at surfaces. *ASM News*. **58**: 202-207.
- Marszalek, D.S., Gerchakov, S.M. and Udey, L.R. 1979. Influence of substrate composition on marine microfouling. *Applied Environmental Microbiology*. **38**: 987-995.

- Martin, Y., Williams, C.C. and Wickramasinghe 1989. Atomic force microscope mapping and profiling on a sub 100 Angstrom scale. *Journal of Applied Physics*. **61**: 4723-4729.
- Matsuda, H., Osaki, K. and Nitta, I. 1958. Crystal structure of quinuhydrone, C₁₂H₈O₂. *Nippon Kogokukai Bulletin*. **31**: 611-620.
- McEldowney, S. and Fletcher, M. 1986. Variability of the influence of physiochemical factors affecting bacterial adhesion to polystyrene substrata. *Applied Environmental Microbiology*. **52**: 460-465.
- Niehof, R.A. and Loeb, G.I. 1973. Molecular fouling of surfaces in seawater. pp. 710-718. In Acker, R.F., Brown, B.F., Depalma, J.R., Iverson, W.P., Eds., *Proceedings of the Third International Congress on Marine Corrosion and Fouling*. Northwestern Press, Evanston, Ill..
- Norde, W. and Lyklema, J. 1979. Thermodynamics of Protein Adsorption. *Journal of Colloid and Interface Science*. **71**: 350-366.
- Olivieri, M.P., Loomis, R.E. and Baier, R.E. 1992. Surface properties of mussel adhesive component films. *Biomaterials*, **13**: 1000-1008.
- Olivieri, M.P., Loomis, R.E., Meyer, A.E. and Baier, R.E. 1990. Surface characterization of mussel adhesive protein films. *Journal of Adhesion Science and Technology*. **4**: 197-204.
- Olivieri, M.P., Rittle, K.H., Tweden, K.S. and Loomis, R.E. 1992. Comparative biophysical study of adsorbed calf serum, fetal bovine serum and mussel adhesive protein films. *Biomaterials*. **13**: 201-208.
- Papov, V.V., Diamond, T.V., Biemann, K. and Waite, J.H. 1995. Hydroxyarginine-containing polyphenolic proteins in the adhesive plaques of the marine mussel *Mytilus edulis*. *Journal of Biological Chemistry*. **270**: 20183-20192.
- Pitman, M.I., Menche, D., Song, E.K., Ben-Yishay, A., Gilbert, D. and Grande, D.A. 1989. The use of adhesives in chondrocyte transplantation surgery: In-vivo studies. *Bulletin of the Hospital for Joint Diseases Orthopedic Institute*. **49**: 213-220.
- Pitt, W.G., Spiegelberg, S.H. and Cooper, S.L. 1987. Adsorption of fibronectin to polyurethane surfaces: Fourier transform infrared spectroscopic studies. In Brash, J.L. and Horbett, T.A., Eds., *Proteins at Interfaces: Physiochemical and Biochemical Studies*. pp. 324-338. American Chemical Society, Washington, D.C..
- Prater, C.B. and Strauser, Y.E. 1994. Tapping ModeTM atomic force microscopy applications to semiconductors. *Application Note*. Digital Instruments Inc., Santa Barbara, CA..

- Pringle, J.H. and Fletcher, M. 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. *Applied Environmental Microbiology*. **45**: 811-817.
- Ratner, B.D. and Castner, D.G. 1994. Electron spectroscopy for chemical analysis. In Vickerman, J.C. and Reed, N.M., Eds., *Surface Analysis-Techniques and Applications*. pp. 163. John Wiley & Sons, Chichester, UK..
- Ratner, B.D. and Castner, D.G. 1994. Advances in X-ray photoelectron spectroscopy instrumentation and methodology: instrument evaluation and new techniques with special reference to biomedical studies. *Colloids and Surfaces B: Biointerfaces*. **2**: 333-345.
- Ratner, B.D., Weathersby, P.K., Hoffman, A.S., Kelly, M.A, and Sharpen, L.H. 1978. Radiation-grafted hydrogels for biomaterials applications as studied by the ESCA technique. *Journal of Applied Polymer Science*. **22**: 643-664.
- Robin, J.B., Picciano, P., Kusleika, R.S., Salazar, J. and Benedict, C. 1988. Preliminary evaluation of the use of mussel adhesive protein in experimental epikeratoplasty. *Archives of Ophthalmology*. **106**: 973-977.
- Rzepecki, L.M. and Waite, J.H. 1991. Dopa proteins: versatile varnishes and adhesives from marine fauna. pp. 119-148. In Scheuer, P.J., ed., *Bioorganic Marine Chemistry*, vol.4. Springer-verlag, Berlin.
- Rzepecki, L.M., Hansen, K.M. and Waite, J.H. 1992. Characterization of a cystein rich polyphenolic protein from the blue mussel *Mytilus edulis* L. *Biological Bulletin* **183**: 123-137.
- Soriaga, M.P. and Hubbard, A.T. 1982. Determination of the orientation of adsorbed molecules at solid-liquid interfaces by thin-layer electrochemistry: Aromatic compounds at platinum electrodes *Journal of the American Chemical Society*. **104**: 2735-2742.
- Sarid, D. 1991. Atomic force microscopy. In Sarid, D., Ed., *Scanning Force Microscopy: With Applications to Electric, Magnetic and Atomic Forces*. Oxford University Press, New York.
- Seah, M.P. and Dench, W.A. 1980. Quantitative electron spectroscopy of surfaces: A standard data base for electron inelastic mean free paths in solids. *Surface and Interface Analysis*. **1**: 2-11.
- Shea, C. and Smith-Somerville, H.E. 1994. The effects of phenotype variability on the adhesion properties of *Deleya marina*. *Biofouling*. **8**: 13-25.
- Taylor, S.W., Ross, M.M., Shabanowitz, J., Hunt, D.F. and Waite, J.H. 1994. trans-2,3-cis-3,4-Dihydroxyproline, a new naturally occurring amino acid, is sixth residue in the tandemly repeated consensus of decapeptides of an adhesive protein from *Mytilus edulis*. *Journal of the American Chemical Society*. **116**: 10803-10804.

- Vargo, T.G., Thompson, P.M., Gerenser, L.G., Valentini, R.F., Aebischer, P., Hook, D.J. and Gardella, J.A. 1992. Monolayer chemical lithography and characterization of fluoropolymer films. *Langmuir*. **8**: 130-134.
- Vitellaro-Zuccarello L. 1980. The collagen gland of *Mytilus galloprovincialis*: an ultrastructural and cytochemical study on secretory granules. *Journal of Ultrastructural Research*. **73**: 135-147.
- Vogler, H. 1982. Theoretical study of the orientation dependence of charge-transfer excitations in quinhydrones. *Zeitschrift fur Naturforschung*. **386**: 1130-1135.
- Waite, J.H. and Benedict, C.V. 1984. Assay of dihydroxyphenylalanine (DOPA) in invertebrate structural proteins. *Methods in Enzymology*. **107**: 397-413.
- Waite, J.H. 1983. Evidence for a repeating 3,4-dihydroxyphenylalanine- and hydroxyproline-containing decapeptide in the adhesive protein of the mussel, *Mytilus edulis* L. *Journal of Biological Chemistry*. **258**: 2911-2915.
- Waite, J.H. 1983. Adhesion in byssally attached bivalves. *Biological Reviews*. **58**: 209.
- Waite, J.H. 1987. Nature's underwater adhesive specialist. *International Journal of Adhesion and Adhesives*. **7**: 9-14.
- Waite, J.H. 1989. Marine adhesive proteins: natural composite thermosets. *International Journal of Biological Macromolecules*. **12**: 139-144.
- Waite, J.H. 1990. The Phylogeny and Chemical Diversity of quinone-tanned glues and varnishes. *Comparative Biochemical Physiology B*. **97**: 19-29.
- Waite, J.H., Housley, T.J. and Tanzer, M.L. 1985. Peptide repeats in a mussel glue protein: theme and variation. *Biochemistry*. **24**: 5010-5014.
- Waite, J.H. and Tanzer, M.L. 1980. The bioadhesive of *Mytilus* byssus: A protein containing L-Dopa. *Biochemical and Biophysical Research Communications*. **96**: 1554-1561.
- Williams, T., Marumo, K., Waite, J.H. and Henkens, R.W. 1989. Mussel glue protein has an open conformation. *Archives of Biochemical Biophysics*. **269**: 415.
- Zhong, Q., Inniss, D., Kjoller, K. and Elings, V.B. 1993. Fractured polymer/silica fiber surface studied by tapping mode atomic force microscopy. *Surface Science Letters*. **290**: 688-692.

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