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

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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by

Ace M. Baty III

A thesis submitted in partial fulfillment of the requirements
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APPROVAL

of a thesis submitted by

Ace M. Baty III

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Dr. Bonnie J. Tyler Chairperson, Graduate Committee	Signature	Nov. 13, 95 Date

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Dr. John T. Sears Head, Major Department	Signature	Nov 15, 1995 Date

Approved for the College of Graduate Studies

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Date November 14, 1995

This work is dedicated to Kristi and Ellie

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

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 (LN_2) 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 LN_2 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 proteinacous 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, Rezania, 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 proteinacous 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).

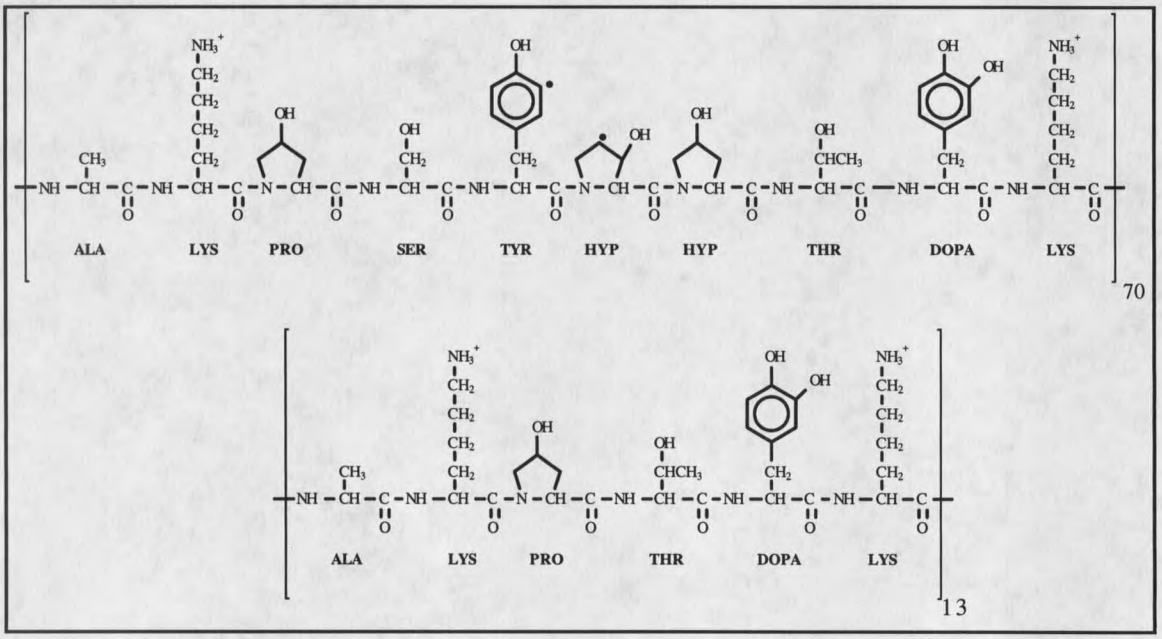

Figure 1. Tandemly repeated deca- and hexa-peptide sequences of MeFP-1, Dots indicate points of optional hydroxylation.

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 scleretization (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 (Rzpecki, 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).

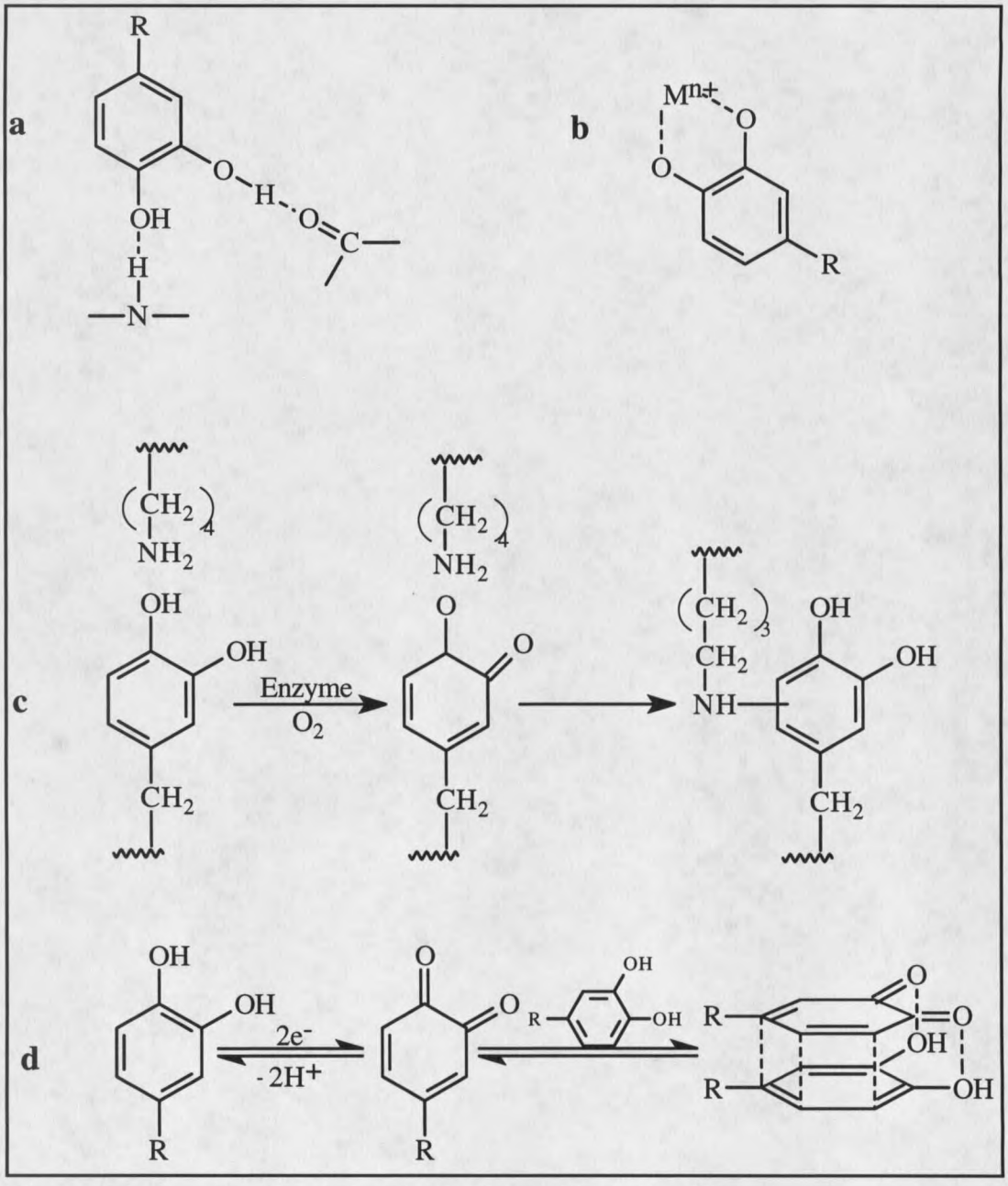

Figure 2. Interactions involving the DOPA residue of MeFP-1.

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 Mode™ is used. In Tapping Mode™ 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 Mode™ 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 Mode™ 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 Mode™. 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 Kα or Al Kα 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)

$$K.E. = h\upsilon - B.E. - \phi_w \qquad (1)$$

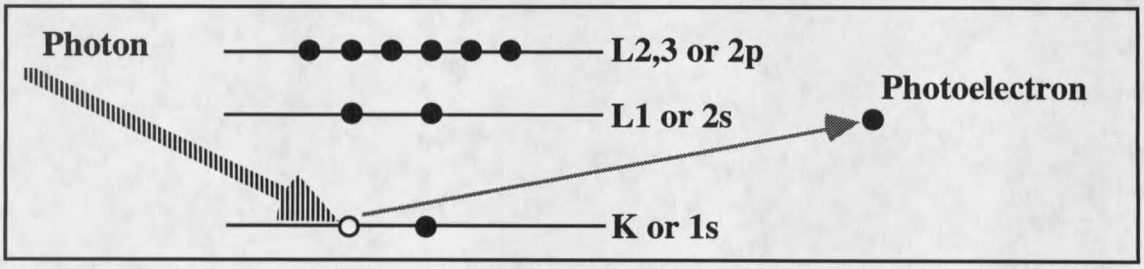

Figure 3: Diagram of the photoelectric process