



Interactions between the pathogenic yeast *Candida albicans* and poly(vinyl chloride)
by Kevin James Siedlecki

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 use of biomedical implants is becoming increasingly widespread in the medical field. The most common cause of device failure is infection of the implant by microorganisms. The pathogenic yeast *Candida albicans* is the third leading cause of these infections trailing only *S. epidermidis*. and *S. aureus*. Interactions between these microorganisms and the implant surfaces are not well-defined, and insight into this area could lead to better material construction to minimize the effects of microbial colonization.

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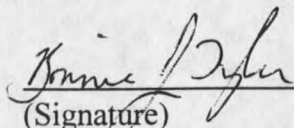
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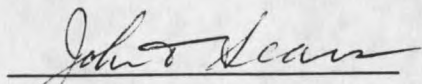
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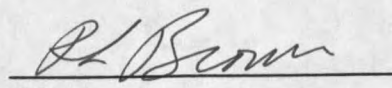
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ABSTRACT

The use of biomedical implants is becoming increasingly widespread in the medical field. The most common cause of device failure is infection of the implant by microorganisms. The pathogenic yeast *Candida albicans* is the third leading cause of these infections trailing only *S. epidermidis* and *S. aureus*. Interactions between these microorganisms and the implant surfaces are not well-defined, and insight into this area could lead to better material construction to minimize the effects of microbial colonization.

Experiments were conducted to study the effects of culture and process variables on *C. albicans* adhesion to poly(vinyl chloride) surfaces. Characterization of the chemical residues left on the polymer surface after removal of adherent yeast cells was also attempted.

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CHAPTER 1

INTRODUCTION

The use of biomaterials for permanent or temporal implantation into the human body is becoming increasingly established.¹ The global market for devices is valued at \$86 billion per annum, with a growth of 7% per year.² These devices include catheters, prosthetic heart valves, pacemakers, and joint replacements. While biomaterials are generally considered safe, problems are often encountered with these implants. The most commonly encountered problem is an infection associated with the material. The incidence of device-related infections ranges from 2.7% to 60%, depending upon the type of device, the underlying disease of the patient, and the criteria used for diagnosis of device-related infection.³ In Canada alone, over 100,000 device-related infections occur each year, costing the health care system over \$135 million.⁴ These infections are usually very serious, and in most cases require removal of the infected device. The results are both costly, inconvenient, and in some cases, life-threatening to the patient.

These devices can easily become colonized by microorganisms which form a biofilm on the surface of the biomaterial.⁵ The biofilm typically consists of the microorganisms as well as a matrix of extracellular polymeric material surrounding the organisms. The infection may then be caused by either the biofilm, detachment of the biofilm cells into the patient, or by planktonic cells. Investigations with pathogenic bacteria have shown that biofilms composed of bacterial pathogens in vitro, have a substantially reduced sensitivity to clinically-important antibiotics compared with cells of the same organism in dispersed form.⁶ Infections typically result in removal and replacement of the infected device. While

this is often an effective treatment, it is costly, time consuming, and dangerous. The ideal solution would be to prevent the organisms from initially adhering to the surface. To accomplish this, a knowledge of the specific mechanism of microbial adhesion to the surface would be valuable. From this, a designer material could theoretically be constructed.

Much work has been accomplished in the area of adhesion of bacterial cells to biomaterials. Although the majority of implant infections are caused by gram-positive bacteria, notably staphylococci, infections due to gram-negative bacteria and fungi tend to be more serious.⁷ Relatively little work has been done on yeast adhesion to surfaces. Among the yeasts, the most important human pathogens are those belonging to the genus *Candida*.⁸ All are opportunistic pathogens causing disease when the host defenses are impaired. The attachment of *Candida albicans* to various biomaterials and host tissues has been deemed an important step in the initiation of both superficial and deep-seated candidiasis.⁹ It is therefore important to elucidate the mechanism of yeast adhesion to biomaterial surfaces.

Previous work has focused primarily on two aspects of yeast cell adhesion to polymer surfaces. The first of these is characterization of environmental effects (such as pH, temperature, media, and cation concentration) on adhesion. Most of the studies have used static adhesion assays that do not accurately portray in vitro situations. The other major focus has been biochemical elucidation of the specific attachment mechanism used by the yeast cell. While these studies have yielded important results, the use of more highly advanced analytical techniques could prove to be a key in elucidating the specific compounds used in the cell-biomaterial interface. This thesis presents results obtained using a flow system which more closely approximates an in vitro situation. It also uses highly advanced

analytical methods to probe the cell-biomaterial interface.

CHAPTER 2

BACKGROUND RESEARCH

2.1 *Candida albicans*

2.1.1 *Candida albicans* Overview

Candida albicans is a pathogenic yeast which causes a variety of infections in people that are characterized by cutaneous, mucosal, or systemic invasion.¹⁰ *C. albicans* is the major etiologic agent of candidiasis and studies show that at least 60% of the *Candida* isolated from sites of infection are of this species.¹¹ Because *C. albicans* is part of the normal human flora, it represents an opportunistic infection.

C. albicans is a dimorphic yeast that grows as both a budding yeast form and a mycelial form. It may exist as either of two phenotypes; opaque or white. Two serotypes have been defined depending on the surface glycoproteins present. One unique aspect of the yeast is its anthropomorphic ability to rapidly change its cell surface in response to new environmental conditions.¹² It has been shown that yeasts grown in media promoting hydrophilicity can change to a hydrophobic cell surface within sixty minutes of a change of media.¹² It has also been shown in laboratory tests that expression of cell surface hydrophobicity results in an increased level of virulence.¹³

The yeast cell can exist in either the budding yeast form, the hyphal form, or the pseudo-hyphal form. The hyphal form is indicated by germ-tube formation from the mother yeast cell. The budding yeast form is round to ovular in nature with a diameter of 3-5 microns. The yeasts reach stationary phase within approximately 18-24 hours of growth in most media.

2.1.2 *Candida albicans* Cell Wall

C. albicans possesses a cell wall consisting of five to eight distinct layers.¹⁴ The total thickness of the cell wall has been estimated at 200-300 nm depending upon growth conditions.¹⁵ The cell wall serves two major purposes; it maintains cell shape and is the point of contact between the cell and its environment. The cell wall is a complex structure composed of ^{甘露聚糖}mannan, ^{葡聚糖}glucan, mannoproteins, chitin, proteins, and a small amount of lipid. Glucan, mannan and mannoproteins constitute at least 80-85% of the cell wall, with the remaining percentage being distributed between proteins (5-15%), lipids (2%), and chitin (0.9-9%).¹⁶ The physical structure of the cell wall is shown in Figure 1.¹⁷

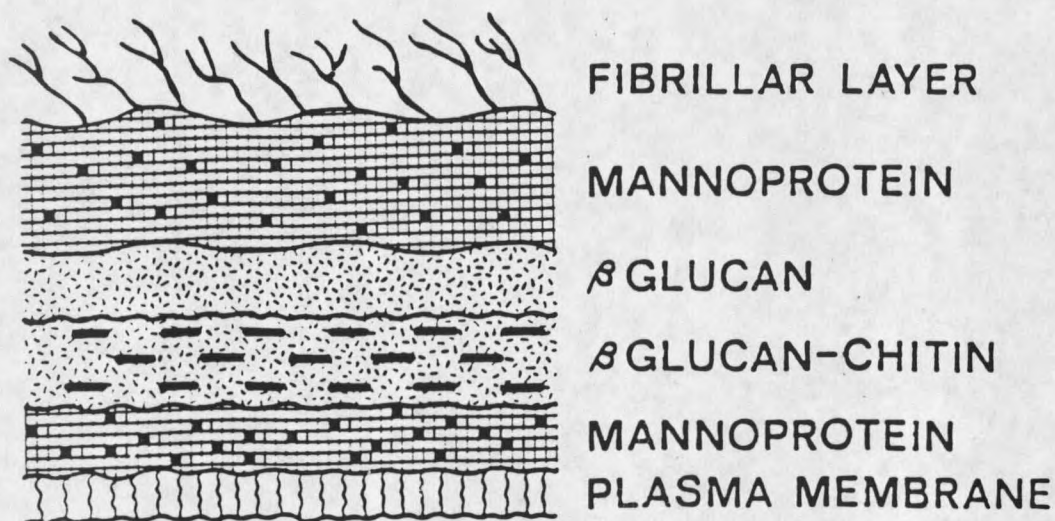


Figure 1: Diagram of *Candida albicans* cell wall structure.

Proteins of the cell wall represent an extraordinary array, the number depending on the growth conditions.¹⁸ Conflicting reports have indicated that proteins either compose a

fibrillar layer projecting from the cell surface, or are equally distributed throughout the cell wall. It is agreed upon, however, that a fibrillar layer on the outermost region of the cell wall appears to be composed primarily of mannoproteins. Since these mannoproteins represent the outermost region of the cell, they may play a key role in the surface-mediated activities of the yeast such as adhesion. It has been shown that growth in a medium with a high sugar concentration will promote the growth of this fibrillar layer.¹⁹

2.1.3 Media Effects

Generally, the pH and chemical composition of the growth medium, as well as ~~接种物~~ inoculum size and incubation temperature, determine the growth form. Hyphal formation is generally enhanced in a medium with a pH higher than 6.5 and a non-fermentable carbon source when grown at high temperatures.²⁰ Growth in an acidic medium using a fermentable carbon source typically promotes blastospore (budding yeasts) production at low incubation temperatures.²¹

2.2 Cellular Attachment to Surfaces

2.2.1 Cellular Attachment: Physical Requirements

Many different environmental and biological factors have been proposed as influencing the adhesion of microbial cells to surfaces. Two main stages of biological adhesion have been recognized; a primary physical attraction stage followed by a secondary biological adhesion stabilization.²² For a cell to become adhered tenaciously to a surface, both of these stages must occur successfully.

There are many proposed mechanisms to explain the initial attachment stage, and a few of these have been regarded as important by nearly every researcher. Almost all researchers agree that the surface properties of both the cell and the biomaterial are important. The attachment of cells to a surface is governed by the physico-chemical properties of both the surface and the cells.²³ 物理化学

Surface free energies must dominate any explanation of the adhesion between different phases which are not mechanically linked.²⁴ The surface free energies of both the cell and the surface must be considered in this. Because surface free energies vary greatly within cellular domains and biomaterials possess a wide range of surface free energies, the role of surface free energies in adhesion varies with the individual microorganism and the biomaterial surface.

Hydrophobic interactions as well as Van der Waals forces must also be considered key elements of the adhesion process. Hydrophobic interactions occur to different extents depending on the degree of hydrophobicity of both the cell and the surface. Studies indicate that hydrophobic forces are exerted at distances as great as 15 nm, and at 8 to 10 nm are 10 to 100 times as great as Van der Waals forces.²⁵ Van der Waals forces may seem negligible, but at the secondary minimum of approximately 10 nm, Van der Waals forces effectively position a particle near the surface.²⁶ Within 1 nm or less of the surface, it is conceivable that short-range chemical interactions (ionic, hydrogen, and covalent bonding) can occur with the extracellular moieties.²⁷

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The process of cellular adhesion is not a well-defined phenomenon. It consists of many different physico-chemical aspects of which all may play either a major or minor role

in the process. An understanding of the driving aspects of this process would help elucidate the specific interactions between a cell and a surface.

2.2.2 Microbial Footprints

The term bacterial “footprint” was used for the first time by Marshall et al. for polymeric material which was left on a surface after the bacterial cells were removed by a shear force.²⁸ The term was used again by researchers who removed bacteria from a surface by proteolytic enzymes. The bacterial “footprint” appears to be one of the mechanisms of initial attachment of cells to surfaces. It is therefore important to elucidate the structure of these adhesive biomolecules. Past work using biochemical assays and advance microscopic techniques has shown the existence of these biomolecules. No reports of “footprint” analysis using advanced surface science techniques have been published. The term “footprint” as used in this thesis refers to the biomolecules tenaciously bound to the surface after removal of adherent yeast cells.

2.2.3 *Candida albicans* Attachment to Surfaces

Of the relatively small amount of data published on yeast adherence to surfaces, the majority has focused on the adherence of the *Candida* species. The most widely documented *Candida* species used has been *C. albicans*. The reason for this is because of its high relevance to medicine. The surfaces used for these studies have included acrylics, poly(vinyl chloride), teflon, and polystyrene. Much of this work has been done looking at the number of adhered cells per area under static attachment conditions. However, one must look at both static and dynamic adhesion, as well as the mechanism of adhesion. Since static fluids exist

rarely in the human body, the attachment of microorganisms in a moving fluid is more ^{/re'livənt/} relevant to looking at implant infections. The mechanism of adhesion has been studied in only a handful of instances, and biochemical assays were the primary method of analysis. This represents a good starting point to expand the current research base by using a dynamic flowing system, as well as highly advanced surface techniques to probe the cell-biomaterial interface.

As stated earlier, *C. albicans* is a dimorphic fungus, and both the yeast and mycelial ^{/ma'i'si:liə/} forms are usually present in infected tissue. However, in the early stages of attachment to and colonization of surfaces, it is the yeast form which is invariably found. For this reason, the yeast form is used in adhesion assays.²⁹ Therefore, the majority of research done in this field has used the yeast rather than the mycelial form of the organism, because germinating ^{/prɒb'l'mæti:k/} cells present unique challenges that are problematic in adherence assays. Those studies will be undertaken after an understanding of a simpler, model system using yeast cell forms is accomplished.

Douglas, et al. has published a number of papers documenting *C. albicans* adherence to acrylic surfaces.^{30,31} Experiments were conducted using a static adhesion assay and a well-defined media. They varied the carbohydrate concentration of the medium between 50 mM and 500 mM using either glucose, galactose, ^{/ˈsju:kraʊs/} sucrose, ^{/mɔ:ltoʊs/} maltose, or ^{/frʌktəʊs/} fructose, as the carbohydrate source. Results showed that organisms grown in medium containing 500 mM-galactose were up to eleven times more adherent than yeasts grown in 50 mM-glucose media.³² The presence of high galactose concentrations is known to augment synthesis of the fibrillar layer, increase mannoprotein content, and enhance hydrophobicity, all

phenomena that may contribute to increased adhesion. They also showed that the presence of divalent cations promote adherence.³³ Another study of the effect of varying the carbohydrate source was conducted by Samaranayake, et. al. in which they showed that cells grown in sucrose or glucose showed enhanced adhesion rates over cells grown in sugar-free media.³⁴ Xylitol and lactose grown yeast cells resembled those grown in the sugar-free control.

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Miyake, et al. found a good correlation between the adherence capacity of *C. albicans* and their affinity for hexadecane.³⁵ These results suggest that hydrophobic interactions play an important role in the adherence of *Candida* species to acrylic surfaces. Results of Klotz, et al. support the work of Miyake, et al. in that there was a linear relationship between the number of cells adhering per unit area and the contact angle of the substratum, i.e. the more hydrophobic the surface, the greater the cell adherence per unit area.³⁶ It was found that *C. albicans* adheres poorly to Pyrex/glass, poly(ethyleneterephthalate) and poly(methylmethacrylate) while it adheres in greater numbers to the more hydrophobic polystyrene and poly(tetrafluoroethylene). This fact does not hold true however for all surfaces since it has been shown that *C. albicans* adheres to PVC in greater numbers than to teflon, even though teflon is a more hydrophobic substrate.³⁷ This group also modified the net surface charge of the yeast and showed that positively-charged yeasts were considerably more adherent than negatively-charged yeasts, indicating the importance of net surface charge on the adherence process.³⁸ However, they also demonstrated that the electrostatic forces were minor relative to the hydrophobic forces.

1'pyrex/ 1'ethylene/ terephthalic acid 对苯二甲酸
派热克斯玻璃 (一种耐热塑料) 乙炔

甲基丙烯酸甲酯 $C_3H_5CO_2CH_3$
聚苯乙烯 四氟乙烯 乙炔

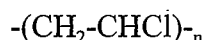
In addition to hydrophobic interactions, others have found that surface free energy

plays an important role in the adherence process. Klotz et. al. found that adherence occurs only when the change in total free energy of the adherence process is negative.³⁹ From a macromolecular view, the adherence of *Candida* species to solid surfaces causes a positive ^{!entropi/}entropy change and also brings about a negative change of free energy.⁴⁰ Minagi, et. al. ^{火酒} found that the closer the surface free energy of the substrate surface and the microorganism, the higher was the probability of adherence.⁴¹ It should be noted that although electrostatic and hydrophobic forces are probably of primary importance in the attachment of *C. albicans* to plastic in vitro, it is likely that additional mechanisms operate in vivo.

All of the above studies were conducted using cells in the yeast form. Only a handful of researchers have investigated germinating cells and their adherence to surfaces. Tronchin, et. al. found that there was a strong correlation between germination and adherence to polystyrene petri dishes.⁴² This suggests that germ tube formation may be accompanied by biochemical changes of the cell wall which lead to the expression of adhesive proteins at the hyphal surfaces. This was later proven by the same group of researchers who found that germ tubes adhering to plastic had developed an additional outermost fibrillar layer containing mannose residues.⁴³ It was also found that these fibrils formed connections with the plastic at the site of cell-substratum contact. This group isolated four proteins left on the polystyrene surface after yeast cell removal. It is likely that one or more of these proteins was involved in the hydrophobic interactions between the cell and the substratum.

2.3 Poly(Vinyl Chloride)

Poly(vinyl chloride) is a simple polymer with the following chemical structure.



Its medical relevance is widely documented and it is primarily used as a material to construct catheters including urinary and intravascular catheters.⁴⁴ Central venous catheters are the intravascular devices most likely to become infected, and most of these are made of PVC. It is an interesting material for use in this study because of its lack of functionalities which may have specific interactions with biological cells.

2.4 Analytical Methods

2.4.1 *Scanning Electron Microscopy*

Scanning electron microscopy (SEM) is perhaps the most widely used high magnification microscopy in the life science field. The SEM experiment yields an image of the sample with up to 100,000 power magnification. Some of the reasons the SEM is so popular in the imaging of surfaces is the large depth of focus, the excellent contrast, and the straightforward preparation of solid specimens.⁴⁵ The major purpose of the SEM is for morphological surface analysis. The two major drawbacks of SEM are that a conducting sample is needed, and the experiment is conducted in a high vacuum meaning that hydrated samples cannot be observed. The first restriction can be overcome by gold sputtering of the sample to help eliminate problems of surface charging.⁴⁶ The second problem, however, cannot be eliminated without the usage of a cryostage or a field-emission SEM.

In an SEM experiment, electrons from a thermionic or field-emission cathode are

accelerated to a voltage of 1-50 kV between a cathode and an anode.⁴⁷ Lenses are then used to focus the beam to a width of 1-10 nm.⁴⁸ The electron beam then irradiates the specimen releasing secondary electrons, backscattered electrons, characteristic x-rays, and several other types of radiation from a small part of the sample.⁴⁹ An image tube detects the emitted secondary electrons and creates an image based upon the positions of the secondary electrons. The image possesses great contrast, good three dimensional focusing, and incredible magnification.

2.4.2 Atomic Force Microscopy

The atomic force microscope (AFM) gives topographic images by scanning a sharp stylus over a surface.⁵⁰ In the AFM, force fields between the probe and the sample are used to guide the probe over the surface.⁵¹ This is considered a near-field microscopy technique that yields a three-dimensional relief of the surface. The image that is obtained reflects the nature of the local interactions between the probe and the surface. Unlike the scanning tunneling microscope, the AFM can obtain images on non-conducting surfaces making it very attractive to the life science field.

Mechanically, the AFM consists of a stylus that is attached to a cantilever. The surface is scanned under the stylus and the interactions between the stylus and the surface cause deflections of the cantilever. The stylus can contact the surface (contact mode), may be vibrated above the surface (non-contact mode) or intermittently contact the surface and oscillate with sufficient amplitude to prevent the tip from being trapped by adhesive forces not directly indicative of the sample (Tapping ModeTM).⁵² Tapping ModeTM has only recently

developed and is showing great promise in the area of adsorbed biological samples. However, the most developed and most commonly used technique is the contact mode technique. In this situation the tip is scanned across the surface of interest. The deflections of the tip due to stylus/sample interactions are measured, and it is this that produces the three-dimensional image. Cantilever deflection can be measured in a number of ways with the optical lever technique being the most common.⁵³ This technique measures the change in angle of light reflected off the back of the cantilever. The angle of light is measured by a position sensitive photodiode. A diagram illustrating the process is shown in Figure 2.⁵⁴ A more complete description of this technique can be found elsewhere.⁵⁵

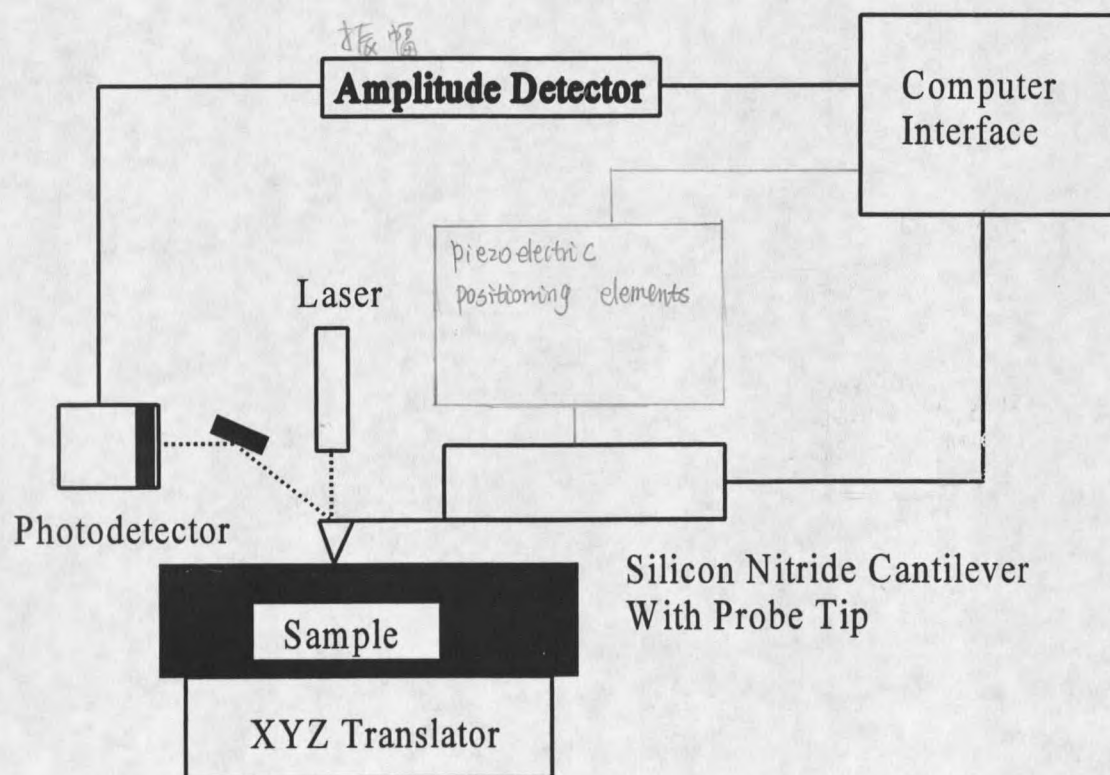


Figure 2: Schematic of the AFM Apparatus.

2.4.3 X-Ray Photoelectron Spectroscopy

X-Ray Photoelectron Spectroscopy (XPS) is perhaps the most widely used surface analytical technique currently available. It is also referred to as Electron Spectroscopy for Chemical Analysis (ESCA). XPS is popular because it is very easy to use, is applicable to a wide number of systems, and provides a wealth of information that is easy to interpret.

The XPS experiment is built around the fact that the electrons surrounding any given element have a certain binding energy to the nucleus of that atom. This binding energy is also affected by the molecules surrounding the particular atom in question. Therefore, a carbon bonded to another carbon will have electrons with a known binding energy. This value will be different if the second carbon is replaced by an oxygen. By measuring the binding energy of an electron, the element that it came from as well as the binding environment of that element can be determined.

In XPS, the surface in question is bombarded by an x-ray source which induces photoelectrons to be emitted. The x-ray source is commonly composed of an aluminum or magnesium anode, which when bombarded by high energy electrons, emits x-rays characteristic of the anode, either AlK_{α} or MgK_{α} x-rays. The kinetic energies of the emitted photoelectrons are measured in the energy analyzer section of the XPS instrument. The energy analyzer is the most crucial part of any XPS instrument. The most common type of energy analyzer used is the spherical sector electrostatic analyzer (SSA). This section disperses photoelectrons based upon their kinetic energies, and these separated photoelectrons are then detected. A diagram of this process is shown in Figure 3. A more detailed account of this process can be found elsewhere.⁵⁶

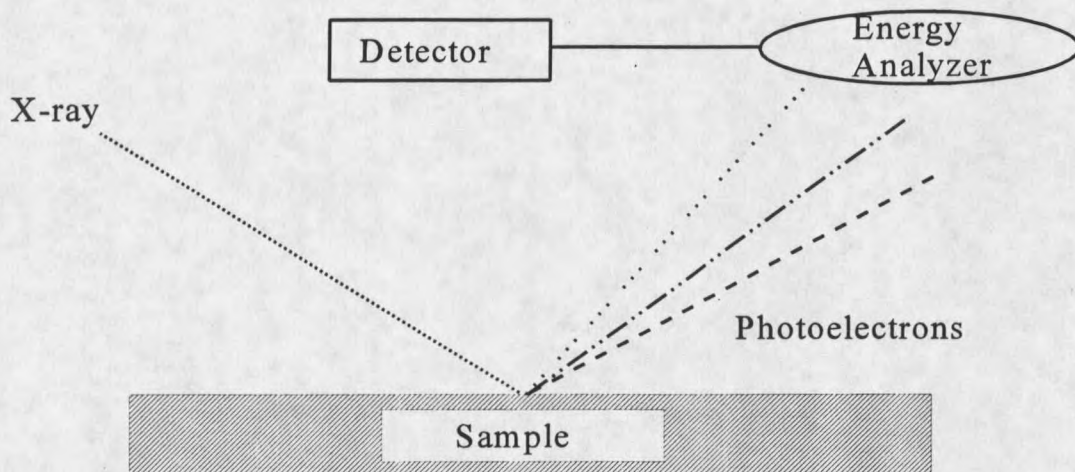


Figure 3: Diagram of the XPS process in which an x-ray irradiates the surface causing the emission of photoelectrons. These photoelectrons are then separated and detected based upon their kinetic energies. This can then be related to binding energy using Equation 1.

A spectrum of the energy of the photoelectrons is then recorded. The binding energy of the photoelectrons is measured by use of the following relationship:

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

where B.E. is the binding energy of the emitted photoelectron; $h\nu$ is the energy of the x-ray source, K.E. is the kinetic energy of the emitted photoelectron, and ϕ is the work function of the XPS system. For photoionization to occur, the binding energy of the electron cannot exceed the energy of x-ray source. The XPS spectrum provides a large amount of information. The most widely used application is the determination of the elemental composition of the surface. The XPS instrument will give these values within $\pm 1\%$. Another important piece of information is achieved by deconvolution of the spectra. Upon

resolving the peak of a given element, it is possible to locate the different binding states of the element in question. An XPS peak may actually be the combination of several gaussian type peaks each specific to a certain binding environment for that element. This is important when trying to determine the structure of an unknown surface. Typical binding energy positions for carbon functionalities are shown in Table 1.

Table 1: Typical Binding Energy Positions for Carbon

Name	Functionality	B.E. (eV)
Hydrocarbon	$\text{C}-\text{H}$ $\text{C}-\text{C}$	285.0
Amine	$\text{C}-\text{N}$	286.0
Alcohol or Ether	$\text{C}-\text{OH}$ $\text{C}-\text{O}-\text{C}$	286.5
Carbon bound to Cl	$\text{C}-\text{Cl}$	286.5
Carbonyl	$\text{C}=\text{O}$	288.0
Amide	$\text{N}-\text{C}=\text{O}$	288.2
Acid Ester	$\text{O}-\text{C}=\text{O}$	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
Carbonate	$\begin{array}{c} \text{O} \\ \\ \text{O}-\text{C}-\text{O} \end{array}$	290.3

2.4.4 Secondary Ion Mass Spectrometry

Secondary ion mass spectrometry (SIMS) is a technique used to determine the molecules present on a surface. This technique has been used for years to characterize the surfaces of conducting samples while its usefulness in analyzing insulating surfaces has just recently become utilized. Another recent area of heightened interest is the use of SIMS in polymer surface analysis.

The principle of SIMS centers around the knowledge that when a surface is bombarded by ions, particles will be emitted from the surface. These particles can be positive ions, negative ions, or neutrals. The secondary ions are then analyzed by a mass analyzer, and a spectrum of the molecules on the surface is obtained. A schematic of the process is shown in Figure 4.

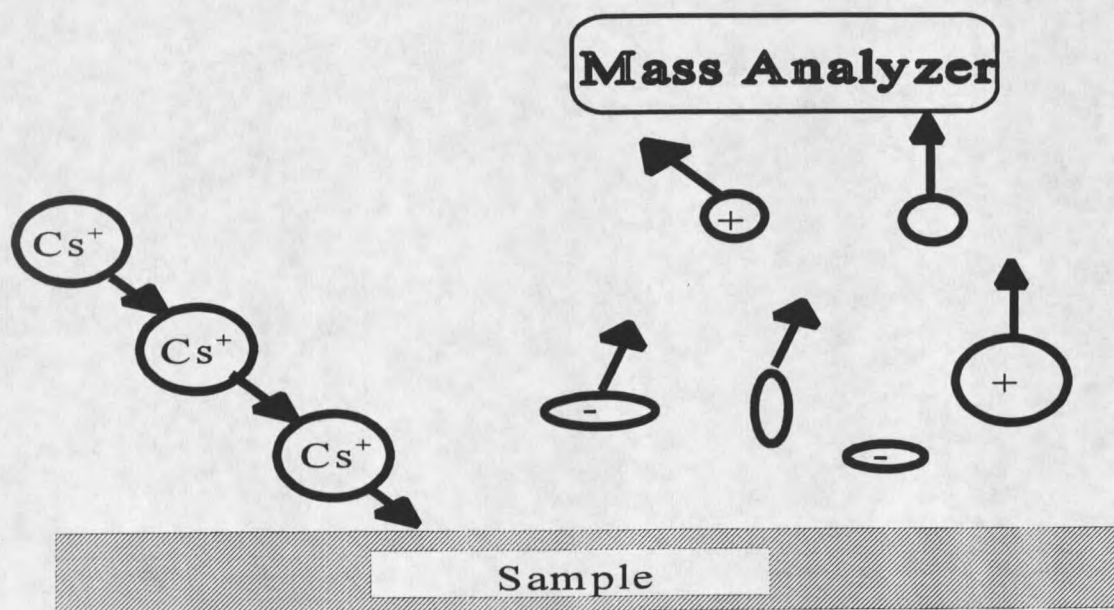


Figure 4: Schematic of the SIMS process where a sample is bombarded by high energy ions resulting in emission of neutrals, positive, and negative ions from the surface. These ions are separated based upon their mass, yielding a mass spectrum of the surface.

The analysis of polymer surfaces is more difficult than conducting surfaces for a number of reasons. The first problem involves charging of the sample. As the sample is irradiated with a positive primary ion beam, the sample begins to accumulate a positive charge. For conducting samples, this is not a problem because the positive charge is quickly dissipated throughout the sample. However, since most polymers are also insulators, this positive charge begins to accumulate and will affect the ions which are created by the initial sputtering process. To account for this, the polymer sample must be flooded by a negatively-charged beam to equalize the charge on the sample. This is typically done by the use of an electron flood gun which is simply a low-energy electron beam. This does not affect the mass spectrum obtained, it simply allows effective charge compensation.

The second major problem associated with analysis of polymer surfaces is damage to the polymer created by the primary ion beam. In static SIMS, the primary ion dose level is kept below 1×10^{13} ions/cm² which allows analysis of the surface without damaging it.⁵⁷ These are typically the two most important criteria that must be met to obtain good spectra of polymer surfaces. Other criteria are given elsewhere and will not be discussed here.⁵⁸

The primary ion source is typically Ga⁺, Cs⁺, or Xe⁺. All of these ion sources have applications which are well suited to their specific characterizations. Cesium and Xenon are very heavy ions which cause a large collision cascade when they impact the surface. These are typically very good for polymer surfaces because of this specific reason. Gallium is a lighter ion, but it can be very finely focused so it is a very good

choice when imaging applications are desired.

The mass analyzer is really the heart of the mass spectrometry unit. The two typical types of mass analyzers used with the SIMS application are a quadrupole mass analyzer and a time of flight mass analyzer. The quadrupole mass analyzer uses DC and RF voltages to selectively allow only ions of a certain mass to complete the path from the ionization chamber to the detector. The voltages are scanned over a wide range of values allowing all the masses to be analyzed. There are two major problems with this type of analyzer. The first problem is that while it is allowing ions of a certain mass to pass, all other masses are ejected leading to a very low detection rate. The second major problem is that typically the quadrupole instrument can only achieve unit resolution meaning that it can only detect the mass to the nearest whole amu and can separate it from the next amu. This means that different compounds with the same nominal mass cannot be distinguished from each other.

The second type of mass analyzer is the time of flight mass analyzer. This system works on the principle that if all ions have the same kinetic energy, the lighter ions will have a higher velocity. Since all ions are extracted from the sample area by the same extraction voltage, all ions should have the same kinetic energy. Because of this, the time for an ion to travel a given distance is directly related to its mass. The time of flight mass analyzer in its simplest form is a long tube of a known length. The detector records the time at which the ion impacts it and relates this to the mass of the ion. The major advantages of this analyzer are that all ions are detected and the mass range has no theoretical limit. In practice, the resolution limit of a time of flight instrument is about

10,000. The mass resolution is calculated by dividing the mass by the separable mass limit. This means that a mass of 10,000 amu can be distinguished from a mass of 10,001 amu. This also means that a mass of 10.000 can be distinguished from a mass of 10.001 amu. This allows molecules of the same nominal mass to be distinguished from each other. The fact that all secondary ions are detected allows for a higher sensitivity since all ionizable molecules on the surface can be detected.

2.4.5 Contact Angle

Another method for obtaining information on the outermost few angstroms of the surface is the use of the contact angle method. An interpretation of the contact angle can often be made if the following assumptions are valid⁵⁹: 1) The surface is rigid and immobile; 2) the surface is highly smooth; 3) the surface is uniform and homogeneous; 4) the liquid surface tension is well-known and constant; 5) the solid surface does not interact with the liquid; and 6) the liquid spreading pressure is zero. If these assumptions are met, the contact angle can yield very good information about the critical surface tension of the sample.

The most common methods for measuring the contact angle are reviewed elsewhere in detail.⁶⁰ The most widely used technique is the measurement of a stationary drop of a known liquid on a surface. The contact angle of this drop may be measured directly with a goniometer, or by the use of trigonometric relationships. The second method, measurements of the drop dimensions, can be easily achieved using little more than a camera and a syringe. This is the method employed in this thesis.

The trigonometric relationships needed to relate dimensional measurements to contact angle are derived elsewhere and will be shown here in final form only.⁶¹ The contact angles are calculated using the equations:

$$\theta = 180^\circ - 2[\tan^{-1}(\frac{2L}{S})] \quad (2)$$

for angles greater than 90° and

$$\theta = \cos^{-1}(\frac{2H}{D} - 1) \quad (3)$$

for angles less than 90° . Variable definitions are as follows: D is the diameter of the droplet; H is the height of the droplet; and θ is the contact angle of the liquid on the sample. The volume of the droplet is not critical as long as it is small enough that gravitational forces may be ignored.

The most popular and widely used method of characterizing the surface properties of polymers has been to use contact angle data to measure the critical surface tension (γ_c). This is achieved by recording the contact angle for a series of homologous liquids on the sample in question. A plot of the surface tension of the pure liquids versus the cosine of the contact angle yields an intercept indicative of the critical surface tension of the sample. The critical surface tension is defined as the surface tension of that liquid which would just totally spread on the solid surface.⁶² The critical surface tension is a good indicator of the properties of the outermost angstroms of the polymer surface. This data is important because yeast cell interactions occur only with the outermost few angstroms of the polymer surface. Surface tension is also proportional to surface free energy which has been

deemed an important variable in cellular adhesion.

2.4.6 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a non-invasive, surface sensitive technique. When coupled with Attenuated Total Reflectance (ATR), it becomes a very powerful tool for the study of biological/surface interactions. Protein adsorption to surfaces has been studied extensively using this technique.^{63,64} Little work on adsorption of microorganisms to surfaces has been published, and that which has been completed has focussed on bacterial attachment to surfaces.

The FTIR/ATR experiment is based upon the principle that an infrared beam is focused onto the face of an internal reflection element (IRE). Typical materials of construction for the IRE are germanium (Ge) or zinc selenide (ZnSe). These materials are chosen because they are optically translucent to the infrared wave. The radiation reflects internally along the length of the IRE. At each reflection, a decaying "evanescent" wave is established at the external surface of the IRE. Materials at the surface within the distance of the evanescent wave absorb infrared energy and attenuate the infrared radiation exiting the IRE towards the detector. The radiation is then detected typically by a Mercury-Cadmium-Telluride (MCT) detector. The detector senses an infrared spectrum which the FTIR unit processes giving rise to a spectrum indicative of the surface of the IRE. The FTIR/ATR experiment produces a quantitative spectra with peak intensities relative to the amount of species on the surface. Many books have been published describing this process in detail.⁶⁵

Much literature has been published on correlating peaks in the FTIR spectrum to chemical species and chemical bonds.⁶⁶ For the study of microorganism attachment, peaks of interest would include contributions from proteins, polysaccharides, lipids, glycoproteins, and possibly other biologically relevant compounds.

This technique possesses great opportunities for probing the cell-substratum interface. By casting a polymer film onto an IRE, the FTIR unit can analyze the sub-micron thick interface between the polymer surface and the cell. It is in this interface that the adhesion mechanism occurs. It therefore has the possibility to be a very powerful in-situ technique.

CHAPTER 3

MATERIALS AND METHODS

3.1 Procedure for Cleaning Glassware

All glassware used in these studies was cleaned using some combination of the following processes. A solution named "base bath" was used as a basic solution to hydroxylate any materials on the glass surface to aid in their removal. Base bath was prepared using the following recipe. (Note: Base bath is very corrosive and direct contact with the skin can be harmful and must be properly washed and treated immediately). Potassium hydroxide pellets were dissolved in approximately 500 ml of nanopure water. Potassium hydroxide pellets were added until the water solution became completely saturated. When a saturated solution of potassium hydroxide/water was obtained, it was slowly added to approximately 2 liters of ethanol until the ethanol became a saturated mixture of potassium hydroxide/water/ethanol. This point is obvious because of a change in solution color from clear to orange. Care should be taken in all steps of this process because all mixing events described here are exothermic, and precautions should be used to avoid the boiling of any solution. The ethanol/water/potassium hydroxide solution is then used as base bath.

Acid cleaning of the glassware was achieved by one of two methods. The simplest and most common method was the use of a no-chromix/sulfuric acid solution. No-chromix ^{科学子} is an inorganic oxidizer and when mixed with sulfuric acid is a metal-free cleaning solution. The second method was achieved by making a solution of "piranya". (Note:

Piranya is very dangerous and can react violently or even explosively with organics. Extreme caution should be taken when this chemical is used).⁶⁷ Piranya is made by adding 30% hydrogen ^{H₂O₂} peroxide to concentrated sulfuric acid in a ratio of 70% acid : 30 % ^{过氧化物} hydrogen peroxide. The solution is then stirred for approximately one minute before usage.

Glassware was cleaned by soaking in base bath for a minimum of 3 hours followed by a rinse with nanopure water. The glass was then placed into the no-chromix/sulfuric acid solution for a minimum of 3 hours. The glass was rinsed, covered with tinfoil, and baked dry at 110-120 °C for 3-24 hours. Glass pieces used as substrata were cleaned using the same procedure for the base bath cleaning. Substrata samples were then rinsed with water and placed into a piranya solution for 30 minutes. The samples were then rinsed with water and dried using a stream of hydrocarbon-free dry nitrogen.

3.2 Preparation of PVC surfaces

Poly(vinyl chloride) samples were made by the spin casting of a solution of PVC dissolved in tetrahydrofuran. This solution was cast onto glass substrata producing a uniform, smooth film of the polymer. The advantages of using a spin cast film such as this are its known composition, its lack of plasticizers and mold-release agents, and its smoothness as well as high reproducibility of the films. The entire process is now described in detail.

A solution of PVC in tetrahydrofuran was prepared by dissolving 2 grams of high molecular weight, plasticizer free poly(vinyl chloride) pellets (Aldrich Chemical) into 100

milliliters of HPLC grade tetrahydrofuran (Aldrich Chemical). The flask containing the solution was covered with tinfoil to minimize light exposure, and the solution was used as 2% PVC in tetrahydrofuran (w/v).

Samples were first prepared by spin coating a 2 weight percent solution of PVC in tetrahydrofuran onto clean, baked glass at 3500 rpm for 2 minutes. While these films had the correct surface chemistry and smoothness, they lacked robustness and quickly delaminated from the glass in water. Because of this, it was chosen to first chemically alter the glass. Dichloromethylsilane was chosen as the reactant. The silane will react with the SiOH groups present on the glass. If the glass is baked, the majority of the surface would be Si groups and would not possess the oxygen necessary for the silane to react with the surface. The silane reacts with the surface to form the following structure:

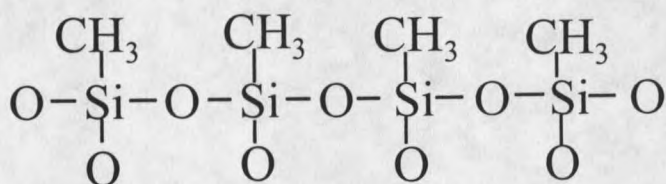


Figure 5: Chemical structure of glass surface modified by reaction with dichloromethylsilane. The methyl groups add a slight hydrophobic nature to the usually hydrophilic glass allowing better contact between this surface and the PVC film.

This surface is more hydrophobic than the clean glass surface thereby allowing the PVC solution to bind to it more tenaciously than to clean glass. By first silanizing the glass, the films became robust enough to remain intact after submersion in aqueous solutions.

Clean glass microscope slides (Clay Adams) were cut into 1 cm x 1 cm squares using a diamond tip pencil. The thickness of these slides was 0.95-1.05 mm. These squares were then rounded and shaped to fit into the recessed wells of the teflon flow cell. This was achieved using either fine or medium emery cloth. Once the glass squares were the correct shape, a reference number was lightly scribed into the upper right hand corner of the glass piece. This number served as both a reference as to the top of the glass piece, as well as a reference to the final outcome of the sample. Glass pieces were then cleaned in base bath for 3 hours with the aid of ultrasonication. The glass was then removed from the base bath, rinsed with nanopure water, and placed in a piranya mixture for 30-60 minutes. The glass was then removed from the acid, once again rinsed with nanopure water, and placed in a clean beaker and dried under a stream of high purity nitrogen. The glass could not be baked dry since physisorbed water on the glass surface is a necessity for the rest of the process.

Once the glass pieces were dried, they were placed number-side up into teflon sample holders. These holders were 1.5 inches x 1.5 inches square with the corners removed. The bottom of the sample holders had been drilled with a 3/16 inch drill bit in 16 spots to allow liquid to freely pass through the sample holders. The sample holders had been cleaned in base bath, no-chromix, then baked before use. The sample holder with the glass pieces was placed into a 150 ml clean glass beaker. Total exposure time of the clean glass to the atmosphere was less than 1 minute typically. The beaker containing the sample holder was then placed into the nitrogen box for the silanization reaction.

Two methods were used for the silanization process. The first method consisted

of a very rigorous process. The second simplified method consisted of a quicker, less labor intensive process. Both processes produced films of similar quality and the simplified method was later adopted as the chosen one. The first method consisted of the following steps. Once the beaker with the samples and the sample holder was in the nitrogen box, 40-60 ml of hexadecane was added to the beaker. The hexadecane (HPLC grade obtained from Sigma Chemical Co.) was triple filtered through an alumina column to remove residual contaminants and water. One pasteur pipet of dichloromethylsilane was then added to the hexadecane yielding a silane concentration of approximately 0.2 M. The solution was stirred with the pasteur pipet for five minutes. The beaker was then covered with tinfoil to prevent evaporation of the hexadecane and the silane. The samples were allowed to soak 24 hours in this solution.

After 24 hours, the teflon sample holder and samples were removed and dip-rinsed in a beaker with chloroform for approximately 1 minute. The samples were then placed into a clean beaker and properly removed from the nitrogen atmosphere.

The sample holder and samples were then carefully removed from the beaker and placed in the top of a soxhlet extractor filled with chloroform. The chloroform was boiled and refluxed over the sample for 30 minutes after complete submersion of the samples. This step was used to remove any unreacted silane from the surface. The samples were then removed from the soxhlet extractor and again placed in a clean beaker. The beaker was covered with tinfoil and placed in an oven at 110-120 °C for 2-3 hours. The baking of the samples removed any residual chloroform from the surface. The samples were then removed from the oven and allowed to cool at room temperature for 12-24 hours.

The spin caster used in this process was a centrifuge converted into a spin caster by removal of the bowl and accompanying shaft. The remaining shaft was thin and approximately 0.5-0.75 cm in diameter. A piece of double sticky tape was placed onto this shaft. The glass sample piece was then placed number side up onto the double sticky tape and gently pressed onto the tape to insure contact. A solution of 2 weight percent PVC in tetrahydrofuran was prepared as described previously. A pasteur pipet was used to place enough of this solution onto the sample to entirely coat the sample creating a large drop on the surface. The sample was spun at 3500 rpm for 2 minutes. The sample was removed from the double sticky tape and gently placed into a clean glass petri dish. The sample was allowed to dry for 24 hours at room temperature to remove any residual solvent. This produced PVC films of high clarity, well-defined composition, and low surface roughness. The only drawback to this technique was its complexity. It did not work well when a high volume of samples were needed quickly. To compensate for this, a simpler method was developed.

The initial stages of this method were the same as described above up to and including the placing of the teflon sample holder and samples into the nitrogen box. At this point, HPLC grade chloroform obtained from Fisher Scientific was used as received. 40-60 ml of chloroform was added to the beaker. One pasteur pipet of dichloromethylsilane (approximately 1 ml) was added to the chloroform and gently mixed for five minutes yielding a silane concentration of approximately 0.2 M. The samples were allowed to soak in the chloroform/dichloromethylsilane solution for 40-120 minutes. The samples were removed and rinsed in 40-60 ml of fresh chloroform for five minutes.

while gently swirling the beaker. The samples were then placed into a beaker containing 50 ml of clean chloroform and soaked for 1-4 hours. At this time the samples were removed and placed into a clean beaker and covered with tinfoil. The beaker was removed from the nitrogen box and baked in the 110-120 °C oven for 2-3 hours. The beaker was then removed and allowed to cool at room temperature for 12-24 hours.

Samples were then spun cast using either the centrifuge described before, or an IEC centrifuge converted into a spin caster by removing the bowl and accompanying shaft. Samples were treated in the same manner as previously described except spun at 4000 rpm for 2 minutes when using the IEC centrifuge. Both centrifuges produced films of similar chemistry, clarity, and surface roughness. The thickness of the films was not determined. Samples were typically used within 1 week of their production.

3.3 Yeast Growth and Culturing

C. albicans strain 1 (CA1) was a clinical isolate received from the collection of Dr. Diane Brawner of Montana State University. It is a serotype B organism. Suspensions of yeast cells in glycerol were prepared and placed into a -70°C freezer for usage as freezer stock. Aliquots of the freezer stock were removed once a month and streaked onto Sabaroud-dextrose agar (SDA) plates. The plates were incubated 48 hours at 35 °C. Isolated colonies were removed from the plates and streaked onto SDA slants. The slants were incubated for 48 hours at 35°C. The slants were then stored at 4°C for one month and used as refrigerator stock.

Yeast Extract-Peptide broth was prepared as follows. Yeast extract at 0.3% (w/v)

and peptone at 1.0% (w/v) were dissolved in nanopure water. A carbon source of either glucose or galactose at 1,3,5, or 9% (w/v) was then added to the water/yeast extract/peptone mixture and thoroughly mixed. 100 milliliters of the solution was placed into 250 ml Erlenmeyer flasks and autoclaved for exactly 15 minutes. Sterilized medium was stored at room temperature until usage. Medium was not stored for a period exceeding three weeks.

Yeast inoculation into the medium was achieved by aseptically removing a loopful of yeast from the refrigerator stock slant. The inoculated medium was then incubated for 24 hours at 35°C in a shaking incubator (160 rpm). After 24 hours of growth, a 0.25 ml aliquot of the medium was removed aseptically and transferred into fresh medium. This flask was incubated again for 24 hours at 35°C and 160 rpm. At the end of the second incubation period, the yeast cells were considered to have grown for 24 hours and will be named "24 hour cells."

Yeast cells were harvested by removing 2-16 ml of the yeast/medium solution and placing it into glass culture tubes. The tubes were centrifuged for 2 minutes to pellet the yeast cells. The supernatant was decanted, and 3 ml of ice-cold PBS was added to each culture tube containing a yeast pellet. The cells were then spun for 2 minutes after which the supernatant was decanted, and the pellet was again washed with cold PBS. This procedure was repeated for a total of three washes. At the end of the third wash, the supernatant was discarded, and the pellet was resuspended in 1-2 ml of cold PBS and mixed with a vortex mixer. A 10 μ l aliquot of this solution was removed, diluted 1:1000 and counted with the use of a hemacytometer and a microscope (450x). Cell counts were recorded and also information about the number of yeast cells per cluster were recorded in some experiments.

Yeast cell/Buffer suspensions were made by placing 100 ml cold PBS into a 125 ml Erlenmeyer flask and adding an amount of the concentrated yeast/buffer solution sufficient to achieve 100 ml of the desired concentration (typically 1×10^6 or 1×10^7 cells/ml). The flask was then placed into a 600 ml beaker containing an ice/water slurry to keep the suspension at 4°C.

3.4 Flow Cell Experiments

Flow cell experiments were conducted with the use of a teflon flow cell. The dimensions of the flow cell are 48 mm long, 12 mm wide, and 0.8 mm deep. Two 1 cm x 1 cm squares were recessed 1 mm into the floor of the flow cell to allow placement of the PVC samples. Flow dynamics in the system were studied by observing the flow pattern created by insertion of a slug dose of crystal violet dye into the flow cell. A flow regime similar of plug flow was observed which correlates well to the low Reynolds number in the system (1.5 to 43.5). Shear rates were calculated by use of the following equations (Equations 4 and 5). Shear rate is defined as the local velocity gradient. Therefore at the surface where the yeast cells adhere, it is the local velocity gradient at the wall. Velocity in laminar flow is described by the following equation:

$$V = V_{\max} \left[1 - \left(\frac{x}{\delta} \right)^2 \right] \quad (4)$$

where x equals the distance from the channel centerline, and δ is one-half the channel thickness. The velocity gradient is then found by taking the derivative of this equation and

using the relationship for laminar flow where the average velocity is one-half the maximum velocity. At the wall, the position x equals one-half the channel thickness. These relationships yield the equation:

$$\frac{dV}{dx} = 4 \frac{V_{avg}}{\delta} \quad (5)$$

This equation describes the shear rate at the point of adherence, and has the units of inverse seconds.

The flow cell is assembled by placing two PVC sample pieces into the two recessed wells of the flow cell. These samples fit snugly into the wells and are even with the teflon surface of the flow cell. The top of the flow cell is completed by placing a #2 glass cover slip (24 x 60 mm) on top of the recessed channel. An aluminum top plate is then screwed onto the flow cell with the use of bolts coming through the top of the plate, and nuts placed into recessed grooves on the underside of the flow cell. For experiments done under high shear rates, the top of the flow cell was modified by adding a poly (vinyl chloride) coverslip of similar dimensions adjacent to the teflon. The glass cover slip was then placed on top of the PVC cover slip, placing the glass between the PVC and the aluminum top plate. This allowed high shear rates to be achieved with minimal leakage in the system. Adherence values did not change with the addition of the PVC cover slip.

The top plate has two unique features. The first is a recessed ovular ring on the inside of the plate allowing placement of an O-ring to securely and evenly press the glass cover slip onto the teflon. This allows a leak-free seal as well as an even distribution of force onto the

cover slip, minimizing the chance that the cover slip will crack during assembly and usage. The second unique feature is a viewing port machined into the top of the flow cell. The rectangular viewing port as well as the beveled edges allow the insertion of a microscope objective into the port to observe the experiment in real time.

The flow cell was constructed of teflon in the interest in minimizing contamination in the system. Teflon is easily cleaned by the usage of base bath followed by cleaning with no-chromix/sulfuric acid solution. The flow cell was cleaned by allowing it to soak a minimum of 3 hours in base bath followed by a rinse with nanopure water. The flow cell was then soaked in no-chromix/sulfuric acid solution for a minimum of 3 hours. Following the acid, a complete rinse with nanopure water was done. The flow cell was then placed into a clean 1000 ml beaker, covered with tinfoil, and placed in a 100-120 °C oven for 3-24 hours. After removal from the oven, the flow cell was placed in a 4 °C refrigerator a minimum of 3 hours and kept in the refrigerator until usage.

The flow system was completed by the addition of a pump and tubing to and from the flow cell. Teflon tubing was chosen because of its high cleanliness and low permeability of air. All teflon tubing used in the experiment was cleaned with base bath, rinsed with nanopure water, and placed in the refrigerator before each experiment. The teflon tubing was connected to the flow cell using stainless steel Swagelok^R fittings. The Swagelok^R fittings were also cleaned in base bath, rinsed with nanopure water, and cooled to 4 °C before usage. A Masterflex^R peristaltic pump with a 1-100 drive rating was used in combination with a three roller, #14 pump head. Tygon laboratory grade size 14 tubing was used in the pump. This section was achieved by placing a 6-8 inch section of Tygon tubing through the pump

rollers. The teflon tubing (1/16" O.D.) was then slid into the Tygon tubing and held firmly by placing 3 cable ties around the Tygon tubing on both the inlet and outlet side of the pump head. This created an air-tight seal and allowed the usage of teflon tubing in a peristaltic pump system. A schematic of the entire system is shown below in Figure 6.

A PBS solution (previously described) was allowed to flow through the flow cell for

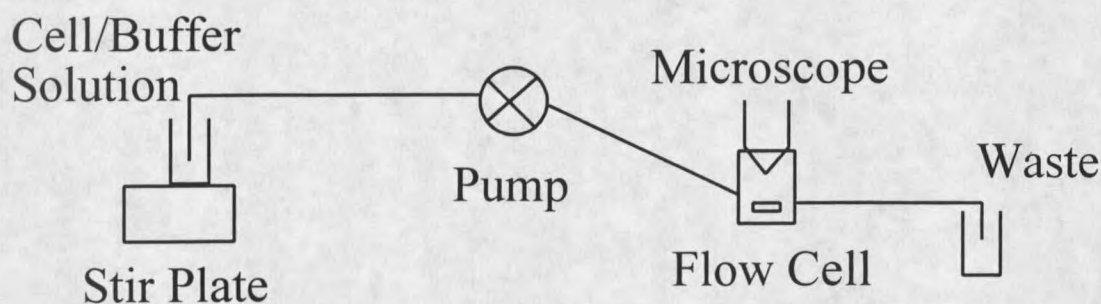


Figure 6: Diagram of system used in all flow experiments.

approximately 5 minutes. This allowed focusing of the microscope, measurement of the flow rate of the system, and elimination of any air bubbles in the system. The yeast/buffer suspension previously described was then flowed through the system for 30 minutes. Microscopic observations were made using an Olympus microscope (Model BH-2) operating in reflected light mode. A lens system of a 20x lens coupled with a 1.5x multiplier was used in addition to a 10x eyepiece yielding a total magnification of 300x. Polarized filters were used to enhance image contrast. Cell counts were taken using an ocular micrometer located in the eyepiece. The size of this grid at 300x was calibrated to be 0.18 mm x 0.18 mm yielding a total area inside the grid of 0.0324 mm². For kinetic studies, cell counts were

taken at various times throughout the 30 minutes. Cell counts were taken for all experiments at 15 and 30 minutes. A minimum of 5 areas were observed using both the first and second PVC samples to achieve this number.

After 30 minutes of flow, a number of different scenarios were explored. Each will now be explained. In all cases, if further flow was used, it was flow of a clean PBS buffer solution containing no yeast cells. If samples were to be created containing the "footprint", two different methods were used. The first method was to instantaneously turn the flow rate up to the pump maximum (approximately 25 ml/min with a 1-100 drive) and vary the flow rate up and down to try to remove cells from the surface. If this method was used, only samples in which >99.99% of adherent cells were removed were used for surface analysis. "Footprint" samples were also created by carefully removing the PVC samples from the flow cell and placing the samples in a clean beaker of PBS buffer solution. This solution was then placed into an ultrasonic bath for 15 minutes. The samples were removed from the buffer, rinsed with water, and observed with the microscope to check for >99.99% cell removal.

For shear study experiments, the flow cell was flushed with buffer for four minutes at the same flow rate as the attachment portion of the experiment. At the end of four minutes, the flow was increased one pump setting. It was allowed to stay there for one minute and was then decreased for one minute to allow counting of the still adherent cells. The flow was then raised one pump setting over the previous highest value and again allowed to remain there for one minute. This procedure was repeated until either significant leakage in the flow cell occurred, or maximum throughput of the pump had been achieved. In most cases, the second scenario occurred before significant leakage was observed. For studies

conducted in which "footprints" or shear rate effects were not of interest, the flow cell was flushed with buffer, disassembled and cleaned.

3.5 Stationary Cell Studies

For chemical analysis of whole cells, the preparation procedure was used as described above and modified in the following manner. Cells were suspended to a concentration of 1×10^9 cells/ml in cold PBS. Cells were then placed onto silver substrata or glass substrata. Silver substrates were prepared by cleaning high purity silver foil by the following procedure. Samples were cleaned by a 2 minute sonication period using the following solvents; hexane, methanol, chloroform, tetrahydrofuran. The samples were then placed for two minutes in concentrated nitric acid. After this they were rinsed with nanopure water to produce a bright white oxide layer. The silver samples were then cooled to 4 °C before usage. Glass samples were prepared by using the base bath/piranya procedure previously described. Samples were then baked at 110-120 °C for 3 hours. Glass samples were also allowed to cool to 4 °C before usage.

An aliquot of 0.25 ml of the yeast/buffer suspension was then placed on the substrata and allowed to settle for 30 minutes. After 30 minutes, the samples were carefully removed and allowed to dry overnight in clean glass petri dishes. Samples were then analyzed within one week of their production. Some samples were created using distilled water instead of buffer solution for surface analysis.

Purified *C. albicans* antigens C6 and H9 were also received from Dr. Brawner. A crude cell surface extract (unpurified) was also received. A detailed account of how these

antigens were isolated, purified, and analyzed is described elsewhere.^{68,69} Purified antigens C6 and H9 were suspended in HPLC grade water at a concentration of 60 mg/ml, and crude extract was suspended in HPLC grade water at a concentration of 100 mg/ml. A 100 μ l aliquot of the solution was then pipetted onto clean glass substrata. This sample was then dried overnight under vacuum before SIMS analysis.

3.6 Analytical Methods

3.6.1 *Scanning Electron Microscopy*

Scanning electron micrographs were produced using the Scanning Electron Microscope at the Image and Chemical Analysis Laboratory at Montana State University. A JEOL 6100 system with a LaB₆ electron source operating at 8.0 kV was used. The samples were first sputter-coated with gold/palladium to help improve conductivity of the insulating samples. Pressure in the analytical chamber during analysis was typically near 1×10^{-6} torr.

3.6.2 *Atomic Force Microscopy*

Atomic Force Micrographs were acquired using a Digital Nanoscope III AFM at the Center for Interfacial Engineering located at the University of Minnesota. A Si₃N₄ triangular cantilever was used in combination with a square pyramid tip. The spring constant of the cantilever was measured to be 0.38 N/m. Either a 350D or 225J scanner was used with a 3-101 optical head. The scanner used was dependent upon the analysis area. Scans were collected at a 2 Hz scanning speed and a resolution of 256 scans/axis. Images were collected

at 5 μm , 1 μm , and 500 nm scan sizes for the PVC and glass substrata. "Footprint" images were collected at near 25 μm scan size, and then the analysis area was decreased as individual "footprints" were analyzed. Scans where salt crystals from the buffer dominated the analysis area were discarded. Images were manipulated by the use of an Auto Flatten feature and a low pass filter to enhance image contrast and resolution.

3.6.3 X-Ray Photoelectron Spectroscopy

XPS analysis was conducted with either an SSX-100 or a PHI 5600 XPS unit. Footprint analysis was conducted using either instrument, while whole cell, PVC, and conditioning film analysis was conducted with the PHI 5600 model.

Analysis using the SSX-100 unit was conducted by Deborah Leach-Scampavia at the University of Washington. Samples were irradiated with a monochromatized Al $K\alpha$ x-ray source operating at 350 Watts. Spectra was collected at pass energies of either 150 eV (resolution 4) or 25 eV (resolution 1) with a spot size of 600 μm . Charge compensation was accomplished by the use of an electron flood gun impinging on the surface. The binding energy scale was referenced by setting the CH_x peak maximum in the C1s spectrum to 285.0 eV.

Analysis using the PHI 5600 was conducted at the Imaging and Chemical Analysis Laboratory at Montana State University. A monochromatized Al $K\alpha$ source at 1486.6 eV and 350 Watts was used as the X-ray source. Charge compensation was achieved using an electron flood gun and a Molybdenum charge compensating mask. An aperture setting of 4 corresponding to an 800 μm spot size of analysis was used. Pressure in the analytical

chamber was maintained between 1×10^{-9} and 1×10^{-10} torr during analysis. Survey spectra were collected at a pass energy of 46.95 eV, and high resolution spectra were collected with a pass energy of 11.75 eV. Analysis times were held under 20 minutes in all cases to insure that the sample was not damaged.

3.6.4 Secondary Ion Mass Spectrometry

Secondary Ion Mass Spectrometry was conducted using the Charles Evans TRIFT system located at the Imaging and Chemical Analysis Laboratory at Montana State University. Spectra were taken using the Cesium source. Pressure in the analytical chamber was maintained at 1×10^{-8} to 1×10^{-9} torr. An accelerating voltage of near 3500 V was used. Charge compensation was accomplished by the use of an electron flood gun impinging on the sample surface. Spectra were typically collected from 0 - 1000 amu. Typical times of analysis were 1-4 minutes to insure static conditions ($1-9 \times 10^{12}$ ions/cm²). Positive and negative ion spectra were taken.

3.6.5 Contact Angle

Contact angle analysis was done using the sessile drop method previously described in this thesis. It employs the use of a drop of known liquid, a syringe, a sample, an image capturing camera, and a measurement device such as a ruler. A drop of the liquid was placed by syringe on the sample and a picture immediately taken of the drop. A schematic of the system employed is shown in Figure 7.

The image of the drop is then analyzed by use of a ruler to measure both the height

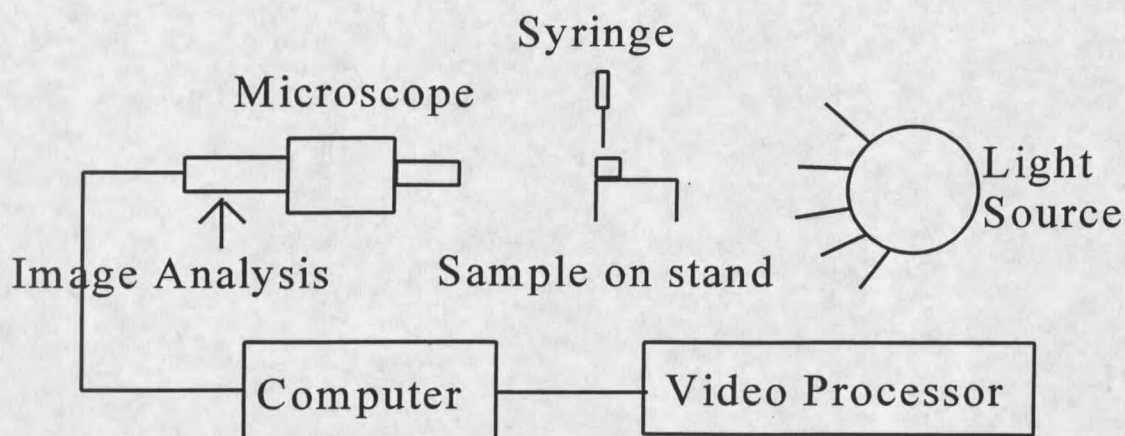


Figure 7: Diagram showing the apparatus employed for contact angle experiments.

of the drop and the diameter of the drop. A contact angle is then calculated by use of equations 2 and 3 given in this text. For the experiments conducted in this thesis, the following liquids were used:

Liquid	Purity	Surface Tension (dynes/cm)
Water	HPLC Grade	72.4
Glycerol	ACS Reagent Grade	64.8
Diiodomethane	HPLC Grade	49.0
Ethylene Glycol	99.8% anhydrous	47.9
Propylene Carbonate	99.7% anhydrous	41.8

Table 2: Table showing liquids used and surface tensions for contact angle experiments.

A 0.3 μl drop of the liquid was placed onto the surface using a 90° beveled syringe. The image of the drop was typically captured within 10 seconds of surface contact. At least

two samples and three drops per sample were used for each liquid. Analysis of contact angle was completed by measuring the dimensions of each drop and using the equations previously given to calculate contact angles.

3.6.6 Fourier Transform Infrared Spectroscopy

The FTIR/ATR flow cell created was a modified version of a HORIZON™ sampling accessory distributed by Harrick Scientific. The HORIZON™ assembly consists of four flat mirrors and two concave mirrors to focus the circular FTIR beam into a spherical beam more conducive to rectangular geometry. The cell and assembly were designed to be used in conjunction with a Nicolet 740 FTIR unit. The requirements of the cell were that it allowed microscopic observation in situ combined with a rectangular geometry IRE. It also had to be easy to clean, and the crystal had to be removable to insure cleanliness, and if needed, the crystal could be coated with polymer.

CHAPTER 4

RESULTS

4.1 Teflon Flow Cell Results

The following set of data was collected using the teflon flow cell system previously described. The purpose was to look at the effect of culture and process variables on yeast cell adhesion.

4.1.1 Carbohydrate Source and Concentration

By growing *C. albicans* cells in a high concentration of carbohydrate, it is believed that the carbohydrate expression of the cell wall is enhanced.⁷⁰ Similarly, growth in a low carbohydrate media promotes expression of the non-carbohydrate portion of the cell wall, or the proteinaceous component of the cell wall. Carbohydrate concentration was therefore varied between 1% and 9% (w/v) using either glucose or galactose.

Figure 8 shows data collected in experiments where glucose or galactose concentrations were varied and yeast cells were adhered at 71 sec^{-1} shear rate. As seen by this figure, glucose-grown cells adhere in greater numbers to the poly(vinyl chloride) surface than cells grown in galactose. This is the opposite of results obtained in static adhesion assays by other researchers who found 9% galactose-grown cells to be up to 10 times as adherent to acrylics as 0.9% glucose-grown cells.

CELL ADHESION VERSUS CARBOHYDRATE CONCENTRATION

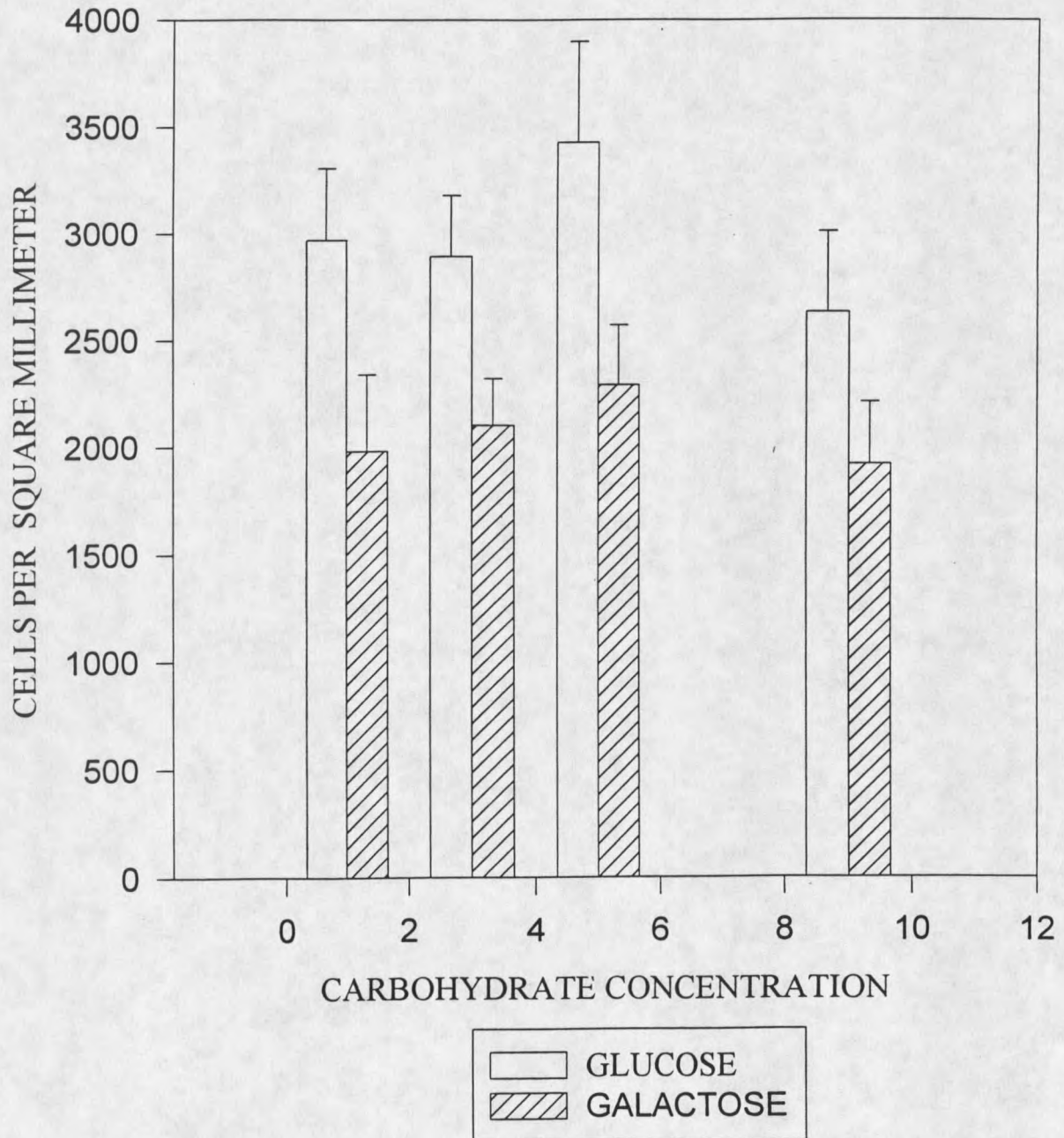


Figure 8: Graph depicting effects of varying carbohydrate sources and concentrations on cell adhesion after 30 minutes. The shear rate during adherence was 71 sec^{-1} .

Another feature to note is the lack of effect of carbohydrate concentration on adherence. A maximum adherence value for glucose appears to be achieved by using 5% carbohydrate, but this difference is not statistically significant in all cases. A maximum adherence value is not found for galactose-grown cells. The data shows that cell wall expression differences between carbohydrate sources are critical to the adhesion process, whereas expression of the proteinaceous or polysaccharide components of the cell wall, as varied by the carbohydrate concentration of the growth media, does not appear to affect yeast cell adhesion.

4.1.2 Kinetic Studies

Kinetic studies were completed by taking cell counts for at least four locations every three minutes. Cell counts were collected and the averages were plotted versus time. A representative graph of the kinetic process is shown in Figure 9.

This graph was obtained using data from two different experiments run under identical conditions. One interesting feature of the graph is the reproducibility found between experiments, typical for all data sets. The most significant aspect of this graph is the linearity of the adhesion process at least to a time period of 30 minutes. This linearity was found for all carbohydrate sources and concentrations. However, this linearity was not consistent for all shear rates as indicated by Figure 10.

The non-first order kinetics at higher shear rates may be explained by the fact that under a high shear field, adherent cells may be more likely to detach during the 30 minutes of experiment time indicated, or as the surface begins to become covered by adherent cells,

KINETICS OF CELL ADHESION 5% GALACTOSE AT 46 INV. SEC

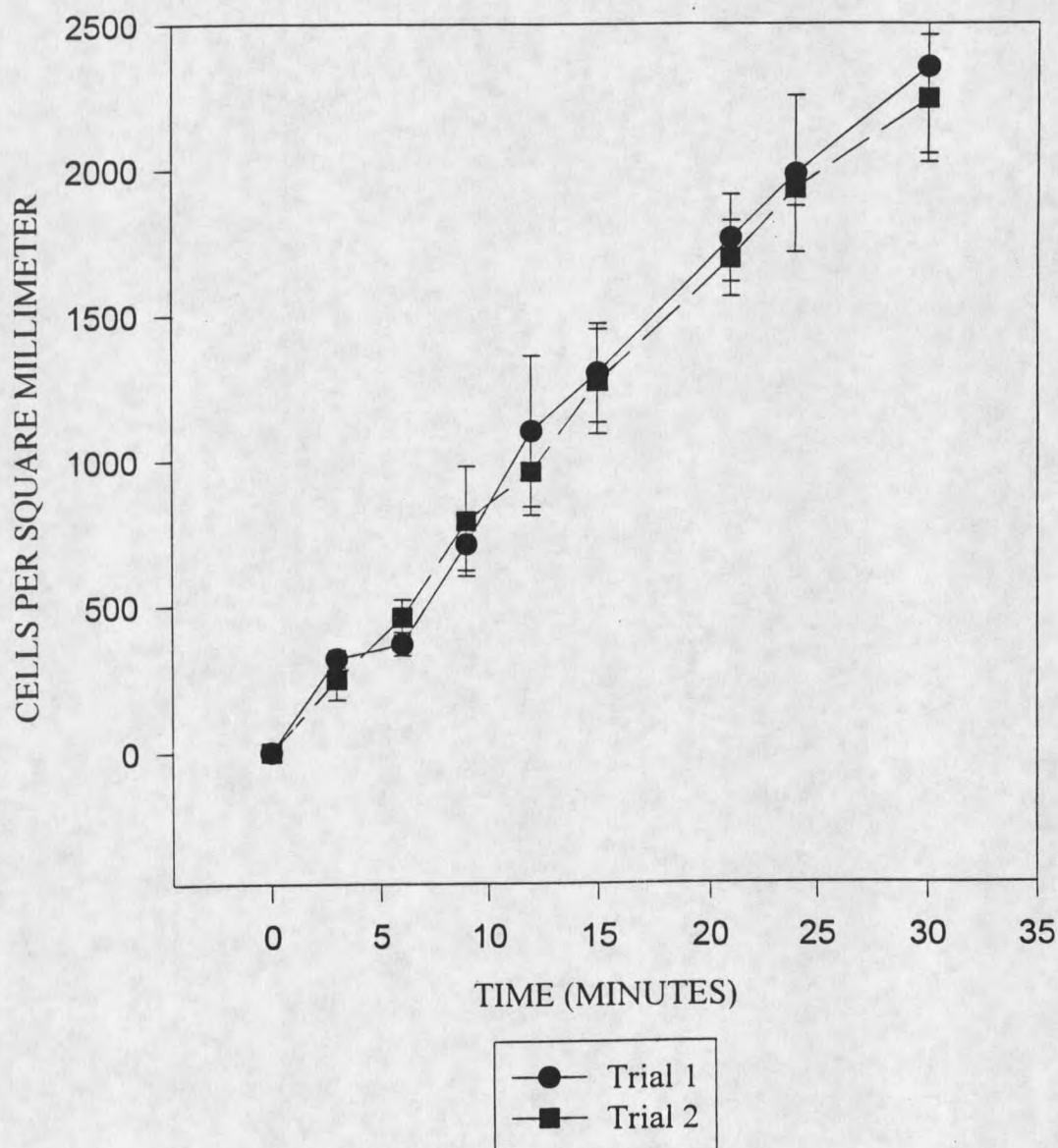


Figure 9: Graph depicting the kinetics of cell adhesion for the first 30 minutes of experiment time. The cells were grown in 5% galactose and the shear rate during attachment was 46 sec^{-1}

KINETICS OF CELL ADHESION 5% GLUCOSE AT 120 INV. SEC

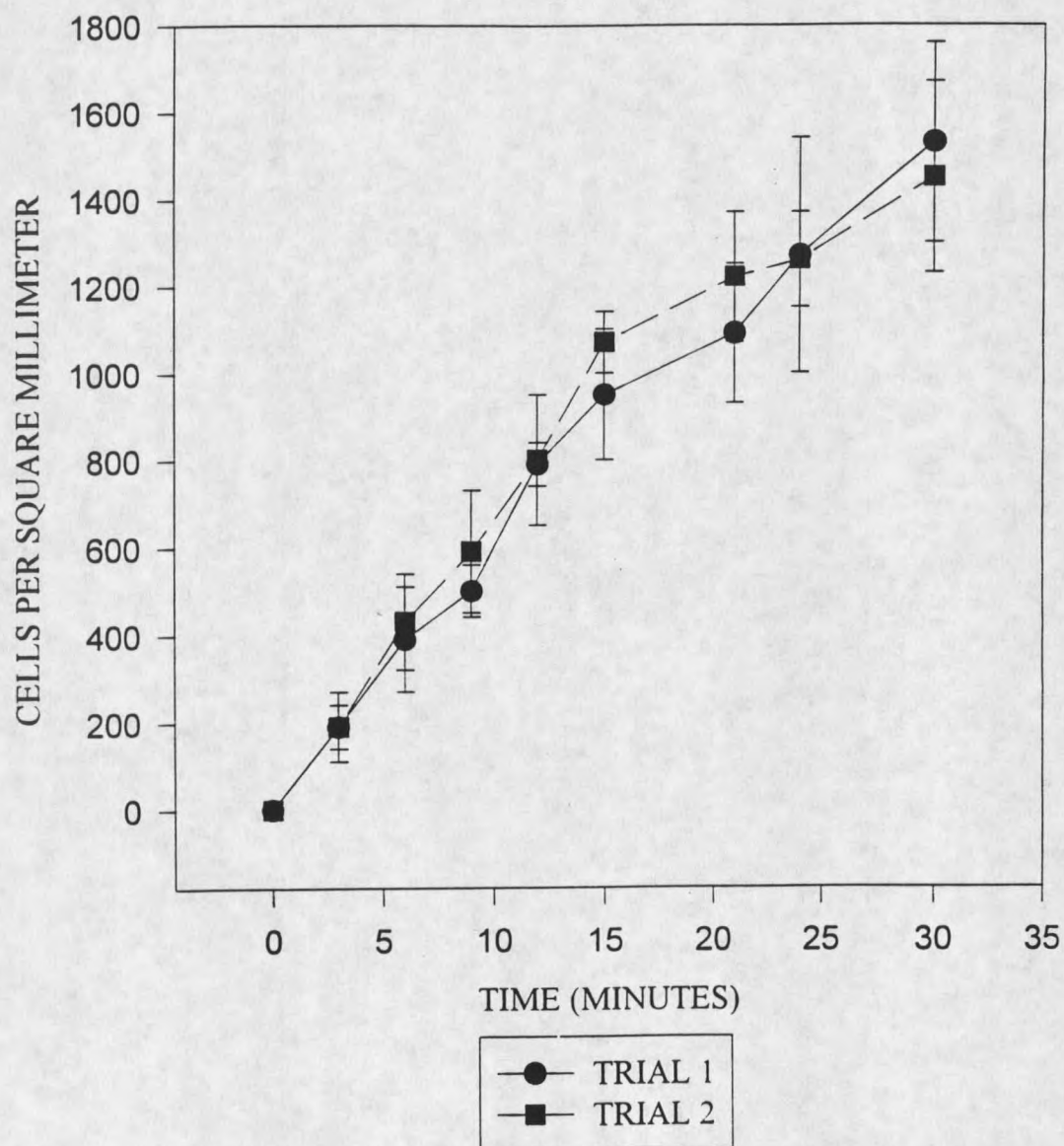


Figure 10: Graph depicting the non-linearity of the cell adhesion process at higher shear rates. Notice that a doubling is not observed in cell counts from 15 to 30 minutes.

the surface is effectively saturated more easily at lower cell concentrations and higher shear rates.

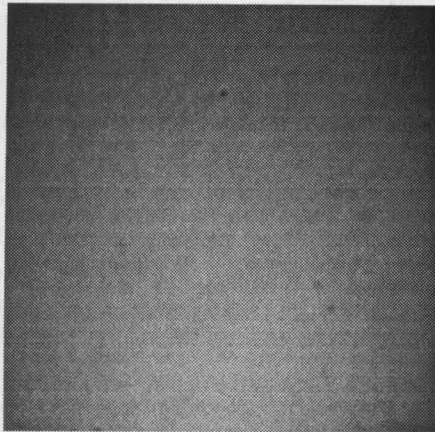
Images of the polymer surface were collected every 6 minutes for both 5% glucose and 5% galactose media sources at a shear rate of 46 sec^{-1} . These images are shown in Figures 11 and 12 respectively.

These images show the adherence process as it occurs. If the images are studied carefully, it is seen that the size and the number of cells per cluster of the glucose-grown cells appear to be different than that of the galactose-grown cells. The glucose-grown cells are larger, and are in clusters of 1-6 cells. The cells in these clusters are all attached by cell walls, indicative of budding phase yeast cells, so it does not appear to be a phenomenon of cell aggregation. Galactose-grown cells are smaller in size and are singlets or doublets of cells. This situation will be discussed later in this thesis.

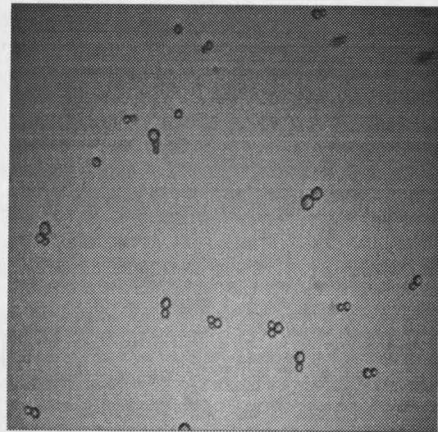
4.1.3 Shear Attachment Effects

Shear attachment effects were studied by conducting a number of experiments under identical growth and adherence conditions, with shear rate being the only variable between experiments. As noted before, shear rate is a function of flow rate and sample geometry and is linearly related to shear force. Shear rate effects were observed for both 3 and 5% glucose- and galactose-grown cells. Figure 13 shows a plot of numbers of adherent yeast versus shear rate after 30 minutes of attachment.

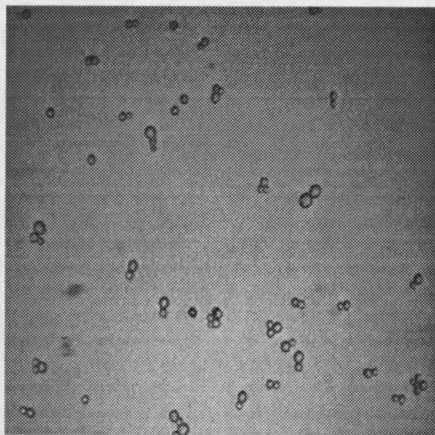
The rate of attachment of glucose-grown cells is more strongly affected by shear rate than the rate of attachment of galactose-grown cells. At 28 sec^{-1} shear rate, glucose-grown



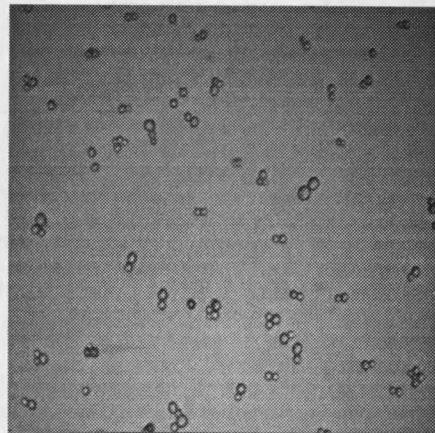
Time = 0 minutes



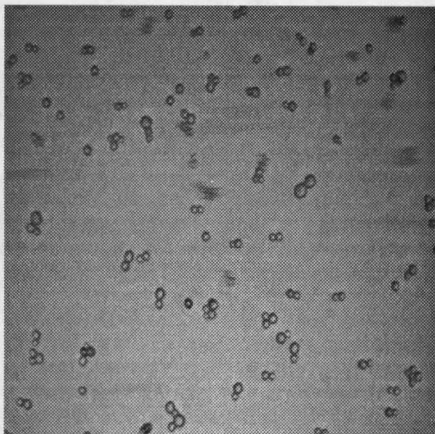
Time = 6 minutes



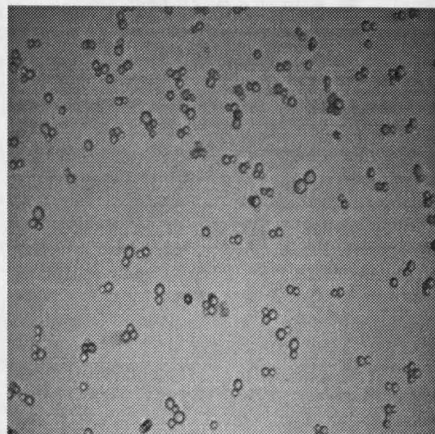
Time = 12 minutes



Time = 18 minutes

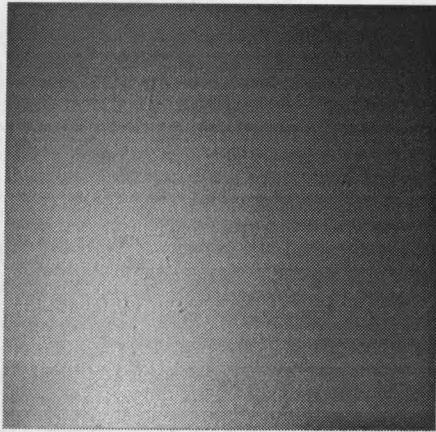


Time = 24 minutes

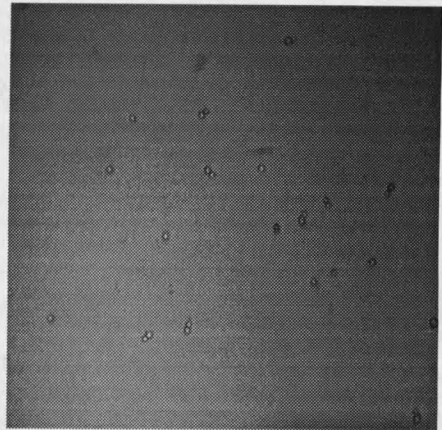


Time = 30 minutes

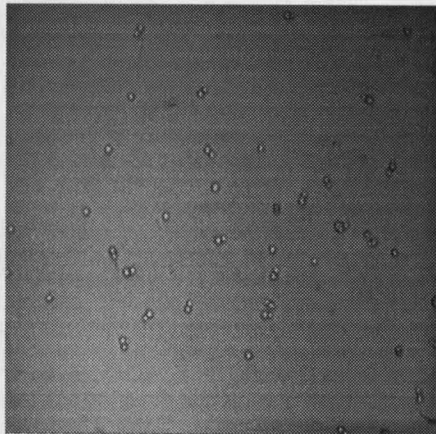
Figure 11: Time lapse images of 5% glucose-grown cells adhering to PVC.



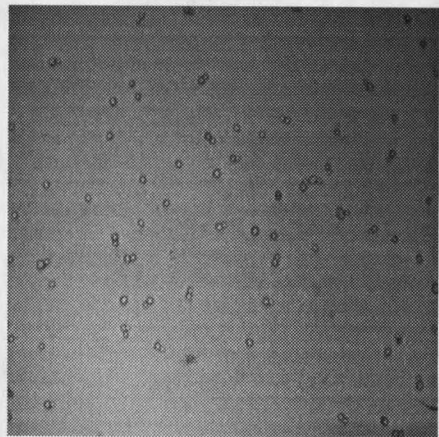
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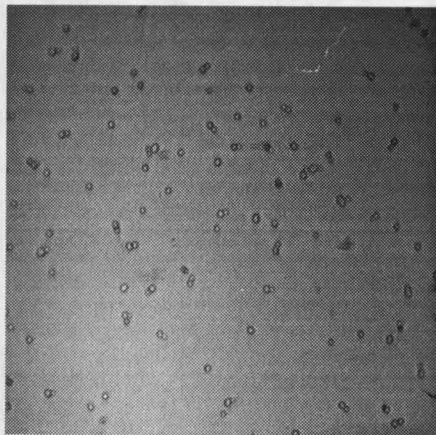
Time = 6 minutes



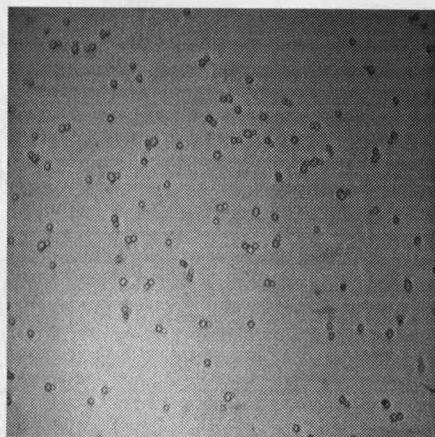
Time = 12 minutes



Time = 18 minutes



Time = 24 minutes



Time = 30 minutes

Figure 12: Time lapse images of 5% galactose-grown cells adhering to PVC.

SHEAR ATTACHMENT EFFECTS 5 % CARBON SOURCE

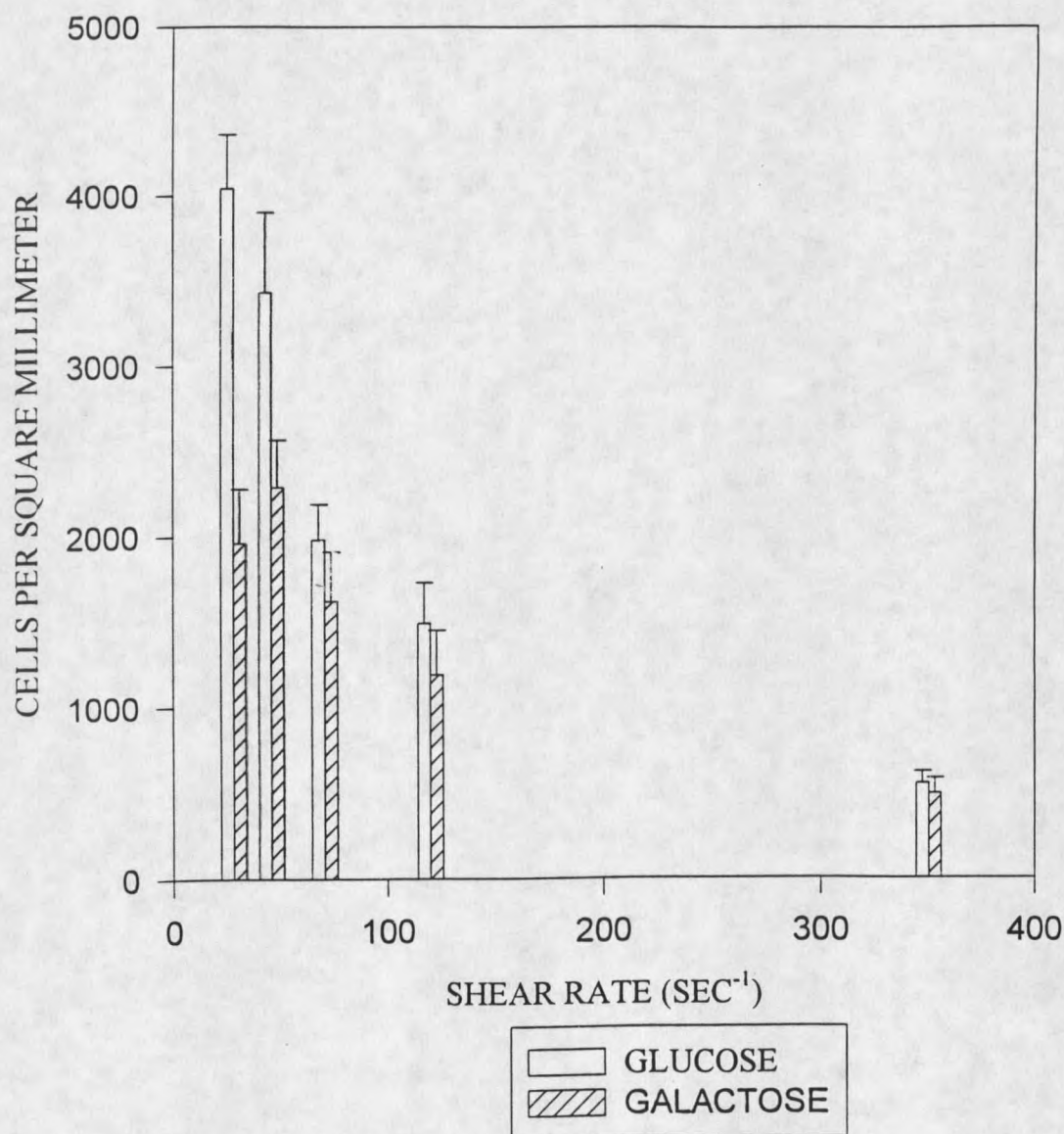


Figure 13: Graph depicting effects of the shear rate at the time of cell attachment. The cells were grown in either 5% glucose or 5% galactose. Note how the difference between glucose grown cells and galactose grown cells decreases as shear rate increases.

cells are nearly twice as adherent as galactose-grown cells. However at 350 sec^{-1} shear rate, there is no statistical difference between the two carbohydrate sources. It therefore appears that galactose-grown cells are less susceptible to shear rate effects than glucose-grown cells. This also proves that a different mechanism may be used for the cells to attach to the surface.

The results of Figure 13 may be misleading upon initial observation. It appears that at 120 sec^{-1} , glucose-grown cells are roughly one-half as adherent as at 28 sec^{-1} . This however, is not true. At a shear rate of 120 sec^{-1} , the flow rate in the system is approximately 4.3 times as great as at 28 sec^{-1} . This means that approximately 4.3 times the number of cells are passing over the PVC surface at 120 sec^{-1} as compared to 28 sec^{-1} , and this results in a reduction of about 2 times in the number of cells per square millimeter. A different way of looking at this effect is to calculate the number of cells passing through the flow cell over the course of an experiment, and correlate this value to the number of cells that adhere to the surface. This calculation does not take into account increased cell transport to the surface at higher shear rates. However, the results would be even more dramatic than those shown in Figure 14.

It is therefore apparent that shear rate effects play a major role in the adherence process, and the mechanism used by galactose-grown cells to adhere is less affected by shear rate than glucose-grown cells.

4.1.4 Shear Detachment Effects

Shear detachment studies were conducted to determine the effect of increased shear rate on adherent cells. Cells were attached to the surface, and after 30 minutes of time, the

SHEAR ATTACHMENT EFFECTS

5% CARBON SOURCE

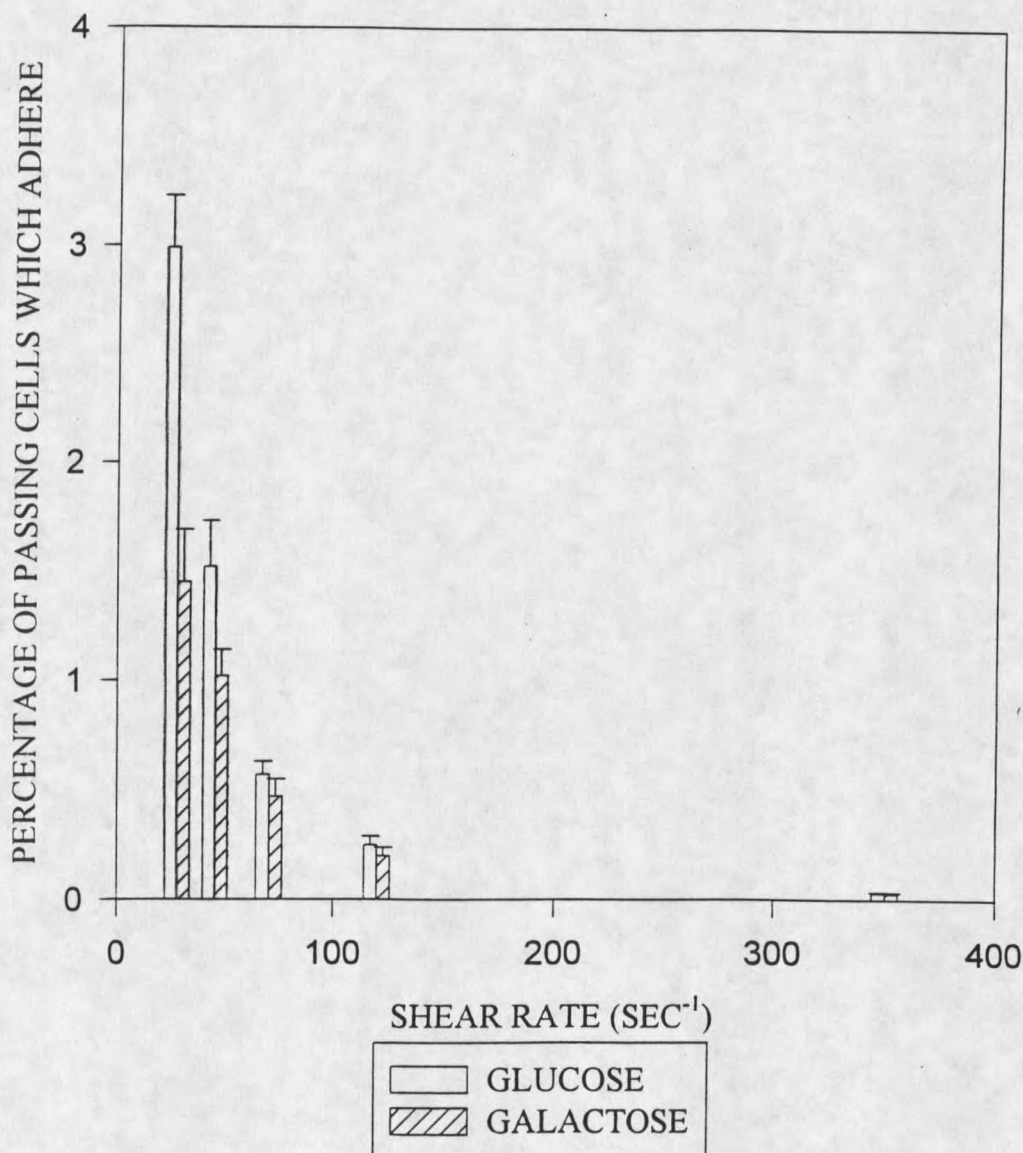


Figure 14: Graph showing the percentage of passing cells that adhere to the PVC surface after 30 minutes. This is calculated by the total number of cells adhered to the surface divided by the total number of cells passing through flow cell during experiment time.

shear rate was incrementally increased to determine its effect on adherence. This was conducted for all experiments and similar results were found throughout. A plot of the results is shown in Figure 15.

As is shown by Figure 15, the strength of attachment appears to be independent of the shear rate during attachment. Yeasts attached at lower shear rates (28 sec^{-1}) seem to be bound as tenaciously to the surface as cells adhered at higher shear rates (120 sec^{-1}). There may be three explanations for this. The first explanation is that the process may be dominated by a free energy driving force. As the cell initially attaches to the surface and displaces bound water, it may very rapidly displace more bound water and adhere very tenaciously to the surface. Depending on the specific energetics involved, this process could occur very rapidly. The second explanation is that the cells may be able to metabolically anchor themselves to the surface during the course of the experiment. This however, is not likely due to the short experimental time and slow metabolic activity of the yeast cells at 4°C . If this was true, some cells adhered during the final 10 minutes of experiment time would detach before metabolism could increase enough for this effect to occur. The second explanation, which seems more plausible, is a mechanical type interaction occurring. If a cell wall has many protrusions, it may take adherence by only one protrusion to anchor the cell initially to the surface at a given shear rate. As the experiment progresses, the shear force on the cell will cause it to wiggle which may allow other protrusions on the surface to attach. This will mechanically anchor the cell to the surface more tenaciously than the initial attachment event did. A diagram of this is shown in Figure 16.

This may also explain the shear effect results on attachment of cells. At higher shear

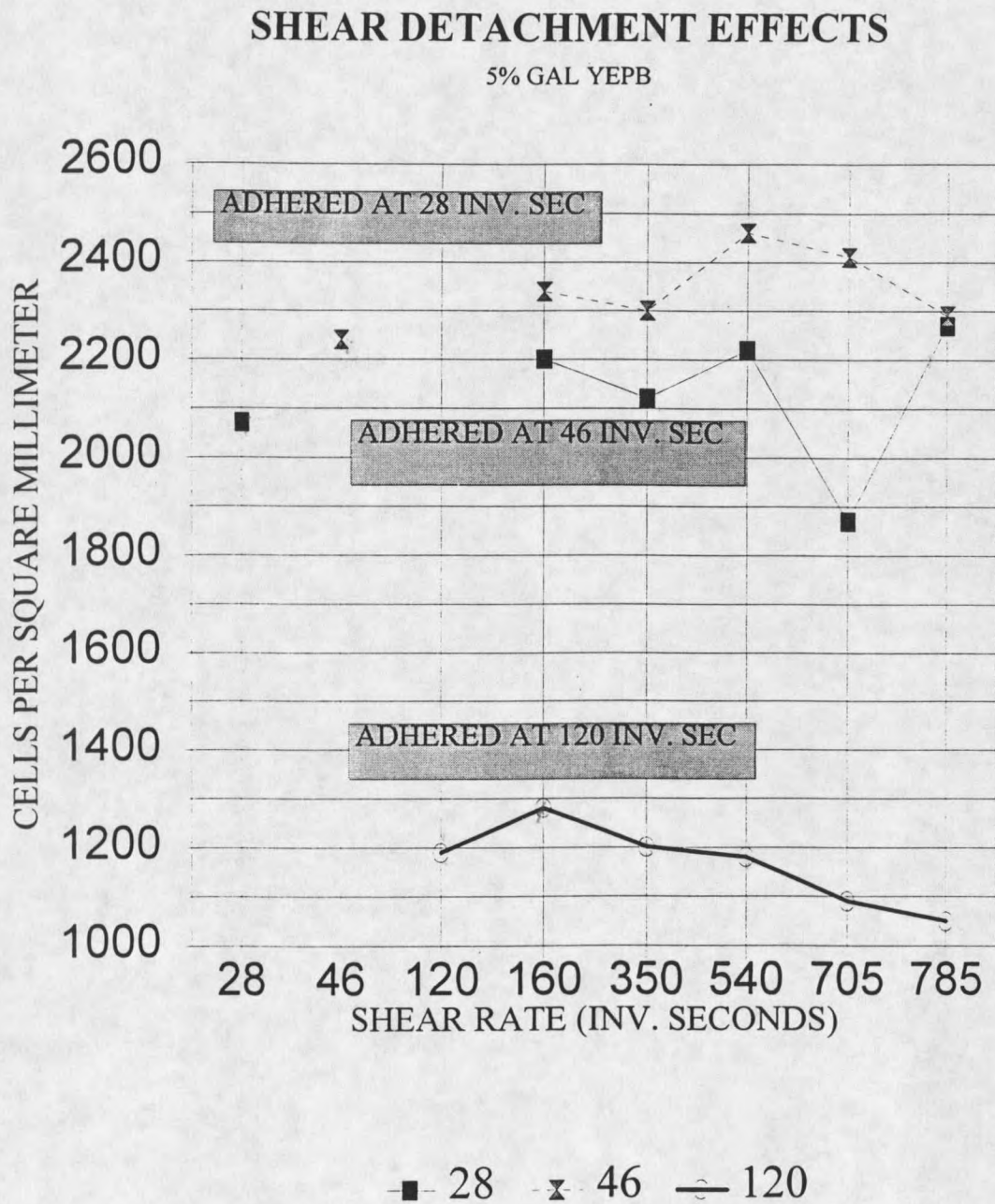


Figure 15: Graph depicting the effect of increased shear rate upon already adherent cells. Notice that the cells are all adhered to the surface very tenaciously regardless of the shear rate of the system during attachment.

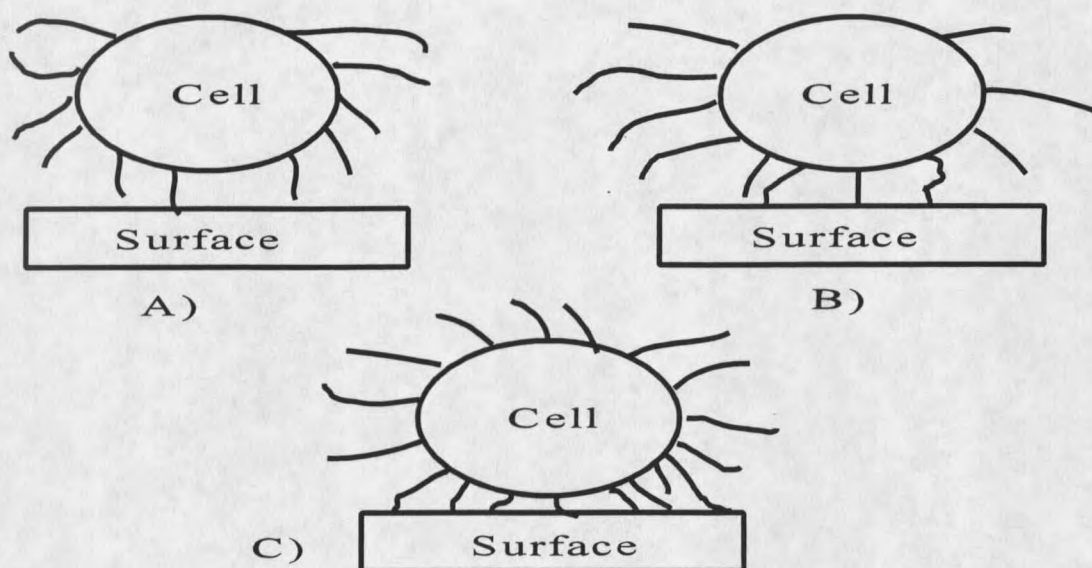


Figure 16: Cartoon of cell adhering to the surface by the use of protrusions from the cell wall. A) Cell initially attaching by use of one extension. B) Cell after a short period of time with 3 mechanical linkages to surface. C) Cell tenaciously bound to the surface after adherence by a number of protrusions.

rates, the strength of one protrusion may not be able to withstand the shear force of the flowing liquid. As the shear rate in the flow cell increases, it may take a greater number of protrusions to initially attach to the surface to allow a cell to adhere. This theory is complimented by the data that indicates that growth in galactose media promotes expression of a fibrillar layer on the cell surface. A fibrillar layer possessing many protrusions, would therefore, have a greater possibility to have multiple attachment sites connect with the surface at any given instant. If glucose-grown cells do not possess this fibrillar layer to as great of an extent, it may be more difficult for multiple sites to attach instantaneously to the polymer surface. This would explain both the tenacity with which adherent cells are linked to the surface, and the fact that glucose-grown cells are more affected during the adherence process by increased shear rates. This theory is further corroborated by the fact that

instantaneously and dramatically increasing the shear rate removes adherent cells. This method does not allow the cell ample time to “wobble” and create new attachments to the surface.

It was also noted that as cells encountered the air-liquid interface, they immediately detached from the surface. This is most likely due to the surface tension of the air-liquid interface which can exert forces on adherent cells up to 1000 times greater than the shear forces used in these studies.

4.1.5 Cell Concentrations, Cluster Size, and Cell Size

Yeast cell concentration after 24 hours of growth in different carbohydrate sources was studied to aid in experiment preparations, as well as to look for metabolic differences related to carbohydrate source utilization. Cell size measurements, as well as numbers of cells per cluster were determined. Table 3 shows the results of these experiments.

MEDIA	CONC. (CELLS/ML)	SD	CELLS PER CLUSTER	SD	CELL SIZE(μ m)	SD
1% GLU	2.35×10^8	0.07×10^8	2.40	1.04		
3% GLU	2.78×10^8	0.37×10^8	2.22	0.80		
5% GLU	2.82×10^8	0.61×10^8	2.41	1.00	5.01	0.90
9% GLU	2.99×10^8	0.73×10^8	2.28	0.89		
1% GAL	2.93×10^8	0.32×10^8	1.50	0.60		
3% GAL	2.73×10^8	0.39×10^8	1.47	0.56		
5% GAL	3.23×10^8	0.22×10^8	1.48	0.55	4.38	0.73
9% GAL	2.87×10^8	0.43×10^8	1.47	0.58		

Table 3: Cell concentrations, cells per cluster, and cell size for a variety of media sources.

As Table 2 indicates, glucose and galactose incorporated into the medium promoted growth to approximately the same cell concentration. However, the average size of the cells are significantly different ($p=0.0001$). Glucose-grown cells are approximately 5 μm in diameter, while galactose-grown cells are roughly 4.4 μm in diameter. The key information provided by this table, however, is the number of cells per cluster for both carbohydrate sources. Glucose-grown cells tend to exist in doublets and triplets, with singlets also present as well as groups of up to 6 cells. Galactose-grown cells exist mainly as singlets with a few doublets, and very limited groups of greater than two cells per cluster. Histograms of the cells per cluster distribution for both carbohydrate sources is shown in Figure 17.

As noted in the section concerning kinetics, it was thought that the number of cells per cluster might affect the adhesion process by a "cooperative" effect. As one glucose cell adhered to the surface, it may have helped approximately 1.3 other cells adhere to the surface. This could, therefore, explain why glucose-grown cells are approximately 1.5 times more adherent at the low shear rates than galactose-grown cells. Image analysis photos were taken of adherent cells at different shear rates to determine if the distribution of cells per cluster changed for adherent cells. A plot of the results of this experiment is shown in Figure 18. As this figure shows, the distribution of cells per cluster does not change for either glucose- or galactose-grown cells as a function of shear rate during attachment. This therefore does not lend credibility to the theory that the size of cell clusters affects the number of adherent yeast cells.

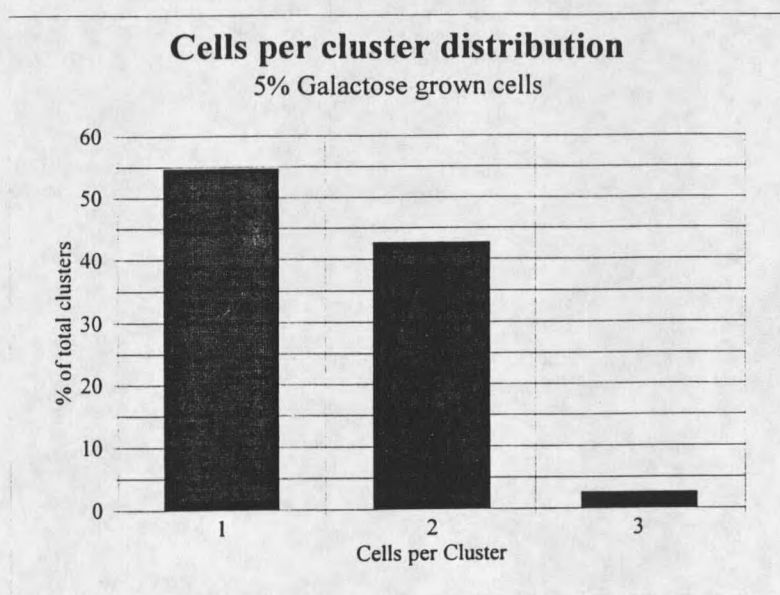
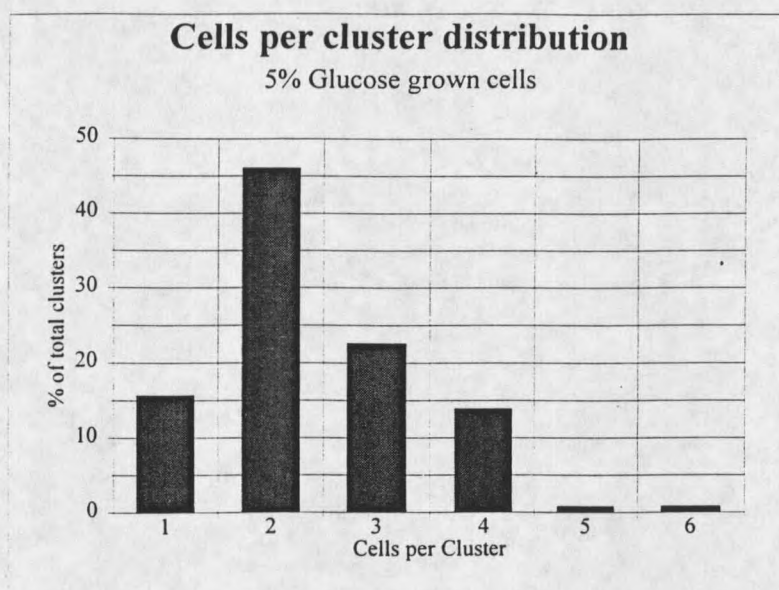


Figure 17: Histograms for distribution of cells per cluster for both 5% glucose and 5% galactose grown cells. Notice the increase in variability for glucose.

CLUSTER SIZE DEPENDENCY ON SHEAR RATE 3% CARBON SOURCE

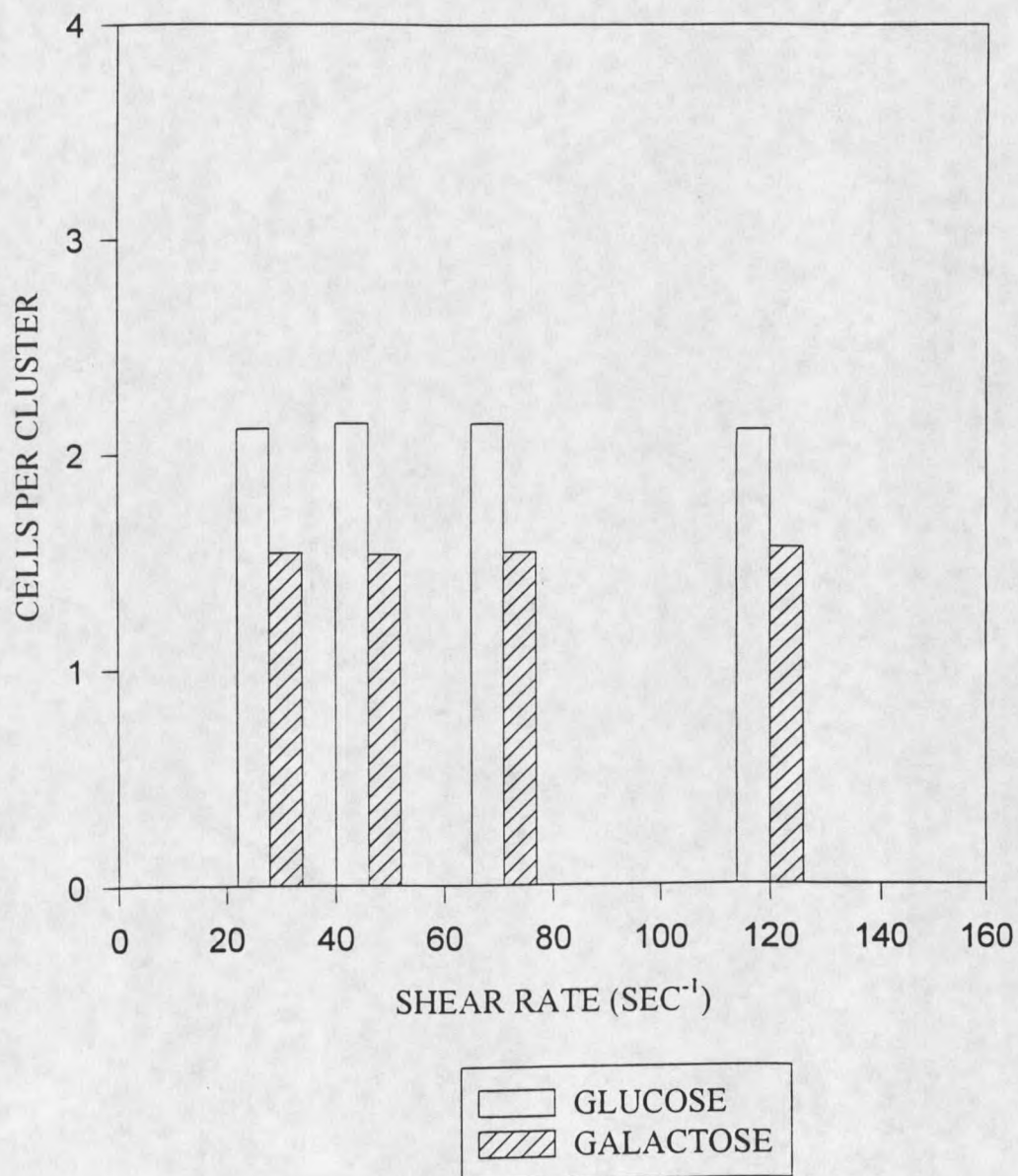


Figure 18: Graph showing the dependence of cells per cluster on shear rate of adherence. No differences were observed from the distribution found for bulk cells.

4.2 Analytical Results

4.2.1 Scanning Electron Microscopy

Scanning electron micrograph images were taken of the poly(vinyl chloride) surface after removal of adherent yeast cells by increased shear force. These images were initially collected to look for the existence of adhesion “footprints”. Results showed the existence of adhesion “footprints”, and yielded topographical information. Figure 19 shows an SEM image of an adhesion “footprint”.

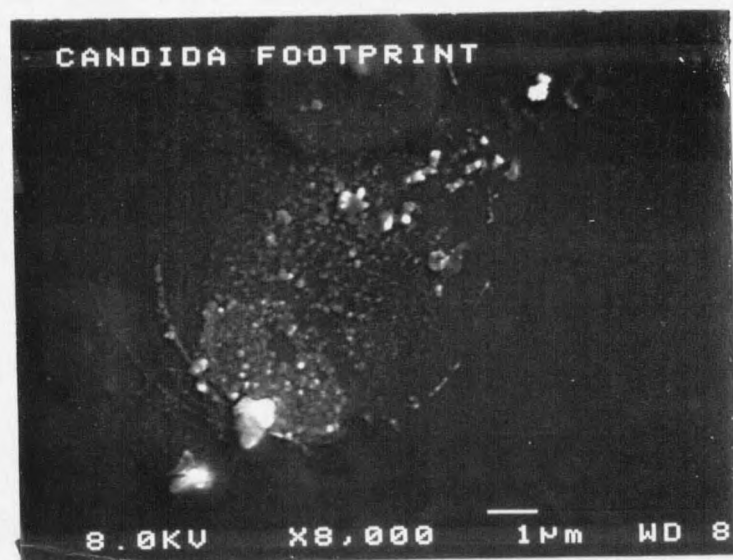


Figure 19: SEM image of “footprint” of *Candida albicans* after adherent yeast cells were removed by increased shear rate.

As seen in the SEM image, the size of the “footprint” is approximately 5 μm in diameter corresponding very closely to the size of the adherent yeast cells. The structure of the “footprint” is structured with a large gathering of biomolecules on one side of the circle, with a scattering of residues in the remaining area. The large protrusion on the lower left

corner of the circle is most likely a salt crystal remaining from the buffer. It should also be noted that not all adherent yeast cells leave a "footprint" upon removal from the surface. This is shown by the fact that about 25% of this surface was covered with adherent yeast cells, while "footprints" cover roughly 1% of the polymer surface. This is most likely due to the fact that cell-polymer interactions are weaker than cell-cell interactions in most cases. However, the residue present in "footprints" is very tenaciously bound to the surface as indicated by the fact that cell-cell bonds had to be broken to remove some of the yeast cell from the surface.

4.2.2 Atomic Force Microscopy

Atomic Force micrographs were obtained for a wide variety of samples. Micrographs were obtained for poly(vinyl chloride) samples to check for continuity and surface roughness. A scan of the clean PVC surface is shown in Figure 20.

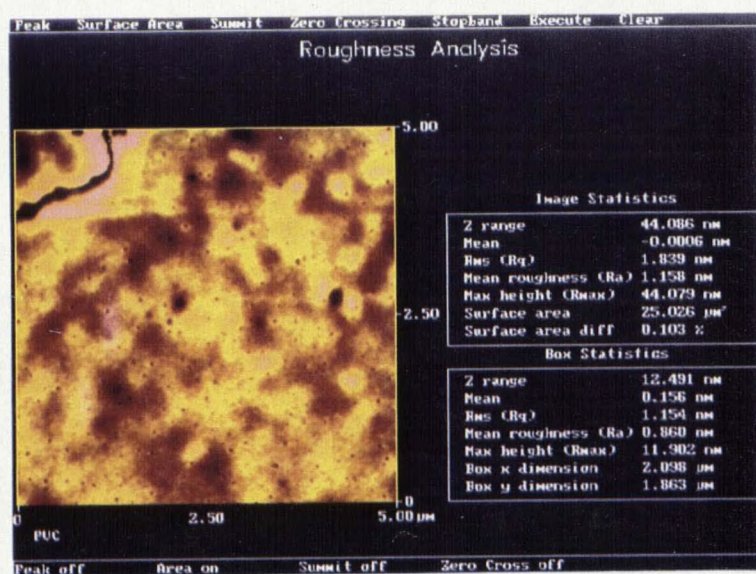


Figure 20: AFM image of poly(vinyl chloride) sample piece. The existence of small dots are microscopic air bubbles in the surface of the polymer.

This sample has an RMS roughness value of 1.158 nm meaning it is very smooth and possesses few topographical variations. It is also shown to be homogeneous and continuous. Because of the high degree of continuity and low surface roughness value, topographical effects on yeast cell adhesion can be neglected.

Micrographs were also obtained on “footprint” samples obtained by shear removal of adherent cells. Both glucose- and galactose-grown cells were studied using this technique. Images were first acquired using a large scan area to find a “footprint”. Scan areas were then decreased to achieve better topographical resolution of the “footprints”.

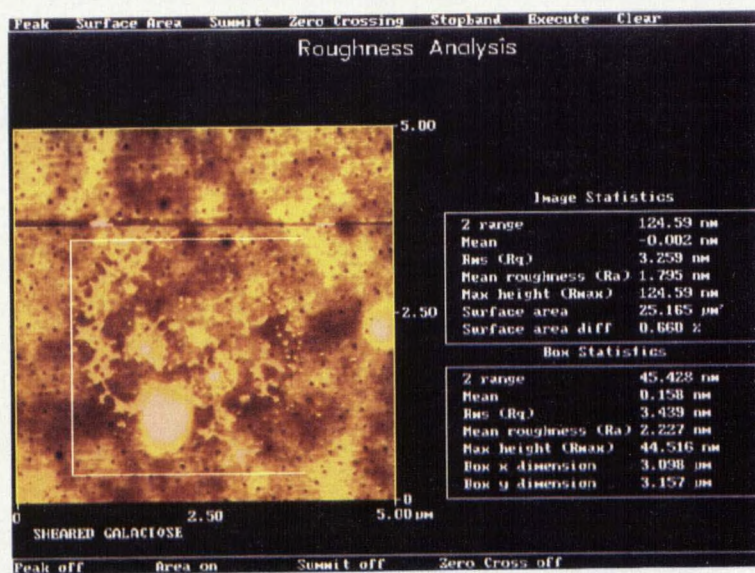


Figure 21: AFM image of poly(vinyl chloride) surface after removal of adherent yeast cells. Notice the size and shape of the “footprint” corresponds closely with the size and shape of the yeast cells.

As shown by the above image, the "footprint" once again has roughly the same dimensions as the adherent yeast cells. As seen in the SEM, there is once again an area of concentrated residues, surrounded by areas of patchy surface coverage. This may be due to the phenomenon of fibrillar attachment as indicated before. Once again, a possible salt crystal is seen in the corner of the "footprint". The "footprint" has a height of roughly 5-10 nm in most spots, with heights of up to 80 nm observed. The RMS roughness value with the "footprint" is 3.54 nm showing increased topography of the sample.

4.2.3 X-Ray Photoelectron Spectroscopy

X-Ray Photoelectron Spectroscopy (XPS) was the technique used to characterize the surfaces used in this thesis based upon their elemental compositions. The polymer (PVC), whole cells, "footprints", and the existence of a conditioning film were all analyzed using this technique.

Clean poly(vinyl chloride) samples were analyzed with XPS to check for thickness as well as surface chemistry of the film. The sampling depth of the XPS technique is generally regarded to be 50-100 Å. A survey spectra of the poly(vinyl chloride) sample is shown in Figure 22.

This figure illustrates that there is no silicon present meaning that the dichloromethylsilane layer and the glass both lie greater than 100 Å from the top of the polymer film. The spectra contains only carbon and chlorine as expected since hydrogen is non-detectable using this technique. From theory the atomic ratio of these two elements should be 2:1 carbon:chlorine. The actual values obtained for a number of polymer samples

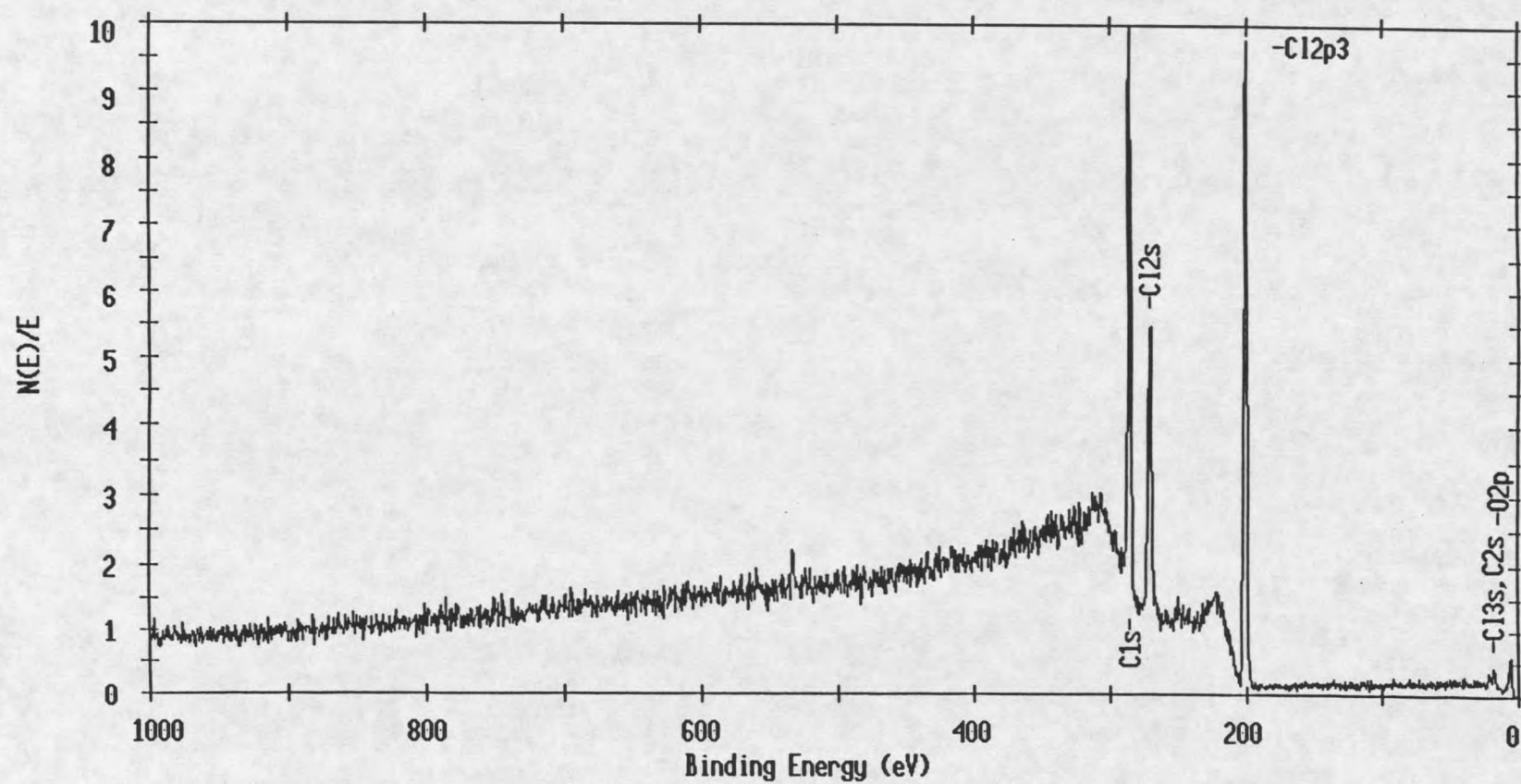


Figure 22: XPS survey spectra of clean poly(vinyl chloride) sample.

were $67.1 \pm 0.3\%$ carbon and $32.9 \pm 0.3\%$ chlorine. Other elements indicative of contaminants or plasticizing agents were not detected.

A high resolution scan of the Carbon 1s region should yield information about the binding environments of the carbon atoms in the sample. Based upon the structure of PVC, two C-C bonds should be seen for each C-Cl bond. Figure 23 shows a high resolution C1s region scan illustrating this 2:1 ratio. Therefore, the polymer used in this study is free of plasticizers, silicon, contaminating agents, and possesses surface chemistry nearly identical to expected values.

Clean PBS buffer was then inoculated with 1×10^6 yeast cells/ml and kept on ice for 30 minutes to simulate an experiment. This buffer was centrifuged, and the supernatant (cell-free), was allowed to contact a clean PVC surface for 30 minutes. Aliquots of the buffer were streaked onto SDA plates to insure the sterility of the buffer. The PVC surface was then analyzed to check for the existence of a proteinaceous conditioning film on the polymer surface. XPS results showed the existence of increased levels of carbon and oxygen, (65.3 % Carbon, 6.2% Oxygen, 28.5% Chlorine) but no nitrogen was detected. A survey scan of this sample is shown in Figure 24.

A high resolution Carbon 1s region scan, showed that the oxygen was present in the form of C-O bonds, probably due to the existence of polysaccharides sloughed off by the wall during the experiment. This scan is shown in Figure 25. It was therefore felt that the experimental set up yielded adhesion results based upon interactions with the polymer, and not yeast-protein interactions.

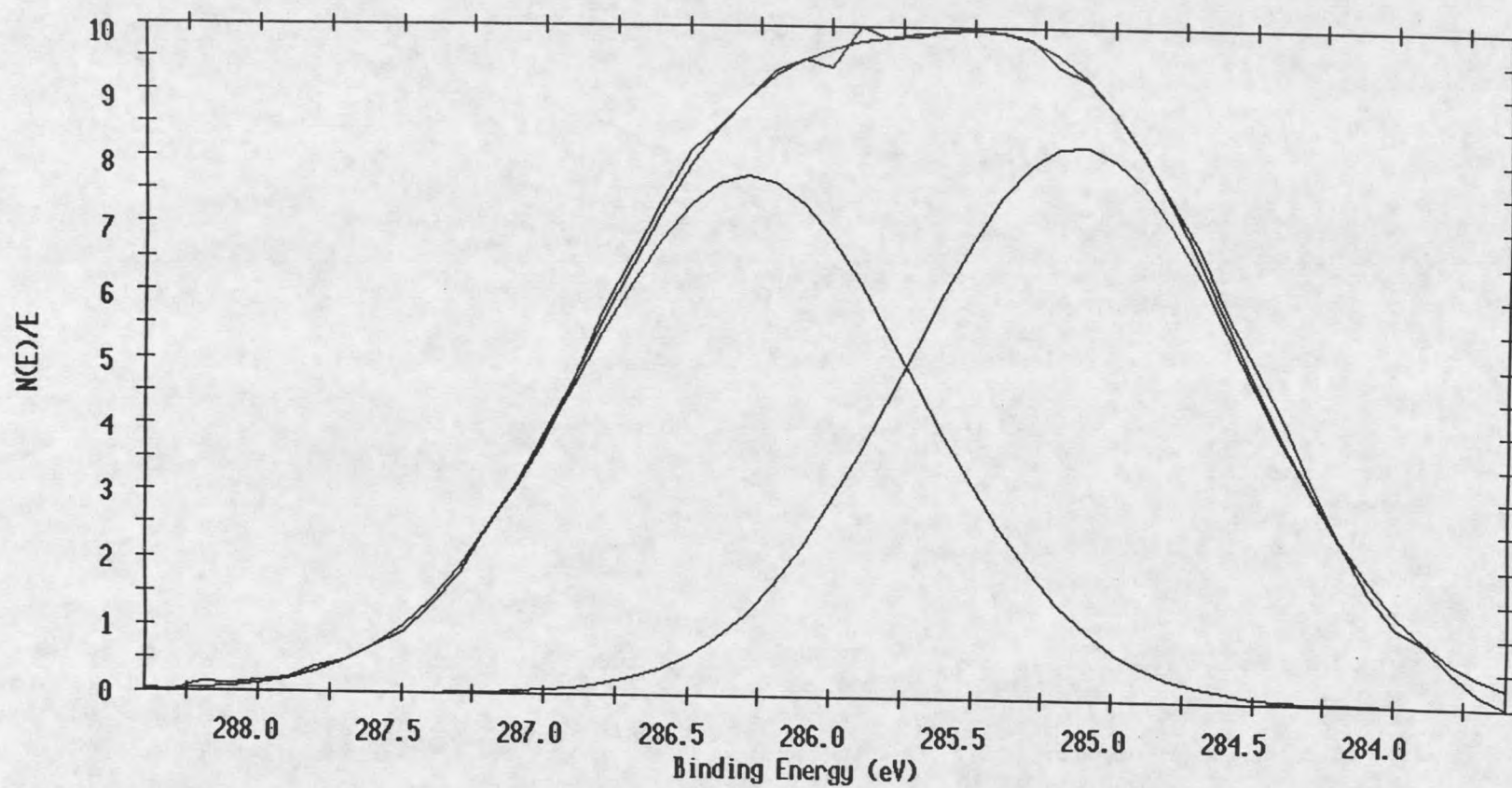


Figure 23: XPS high resolution carbon 1s region showing C-C and C-Cl functionalities.

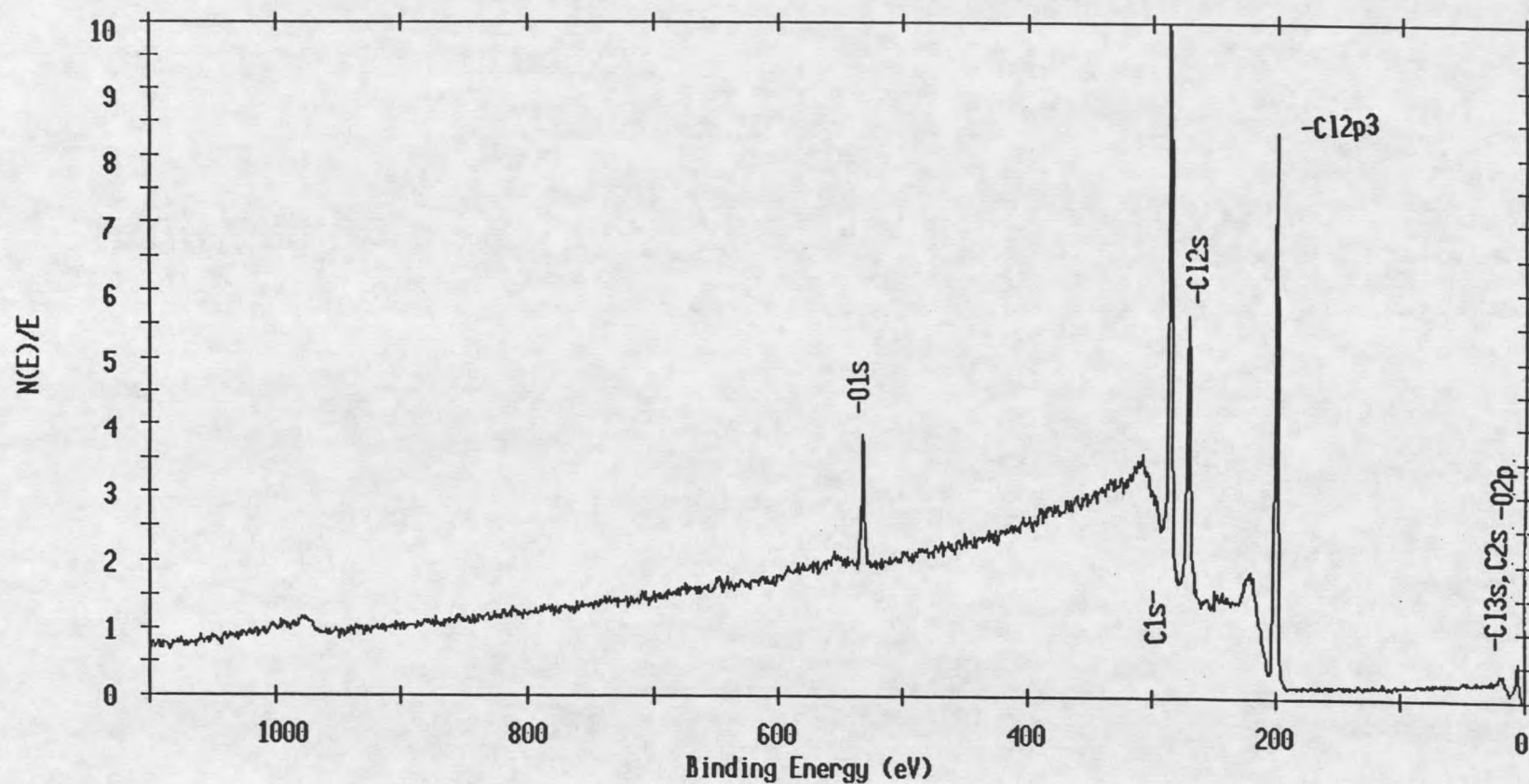


Figure 24: XPS survey scan of PVC surface to look for the existence of a proteinaceous conditioning film. Note that no nitrogen is detected, while oxygen is present.

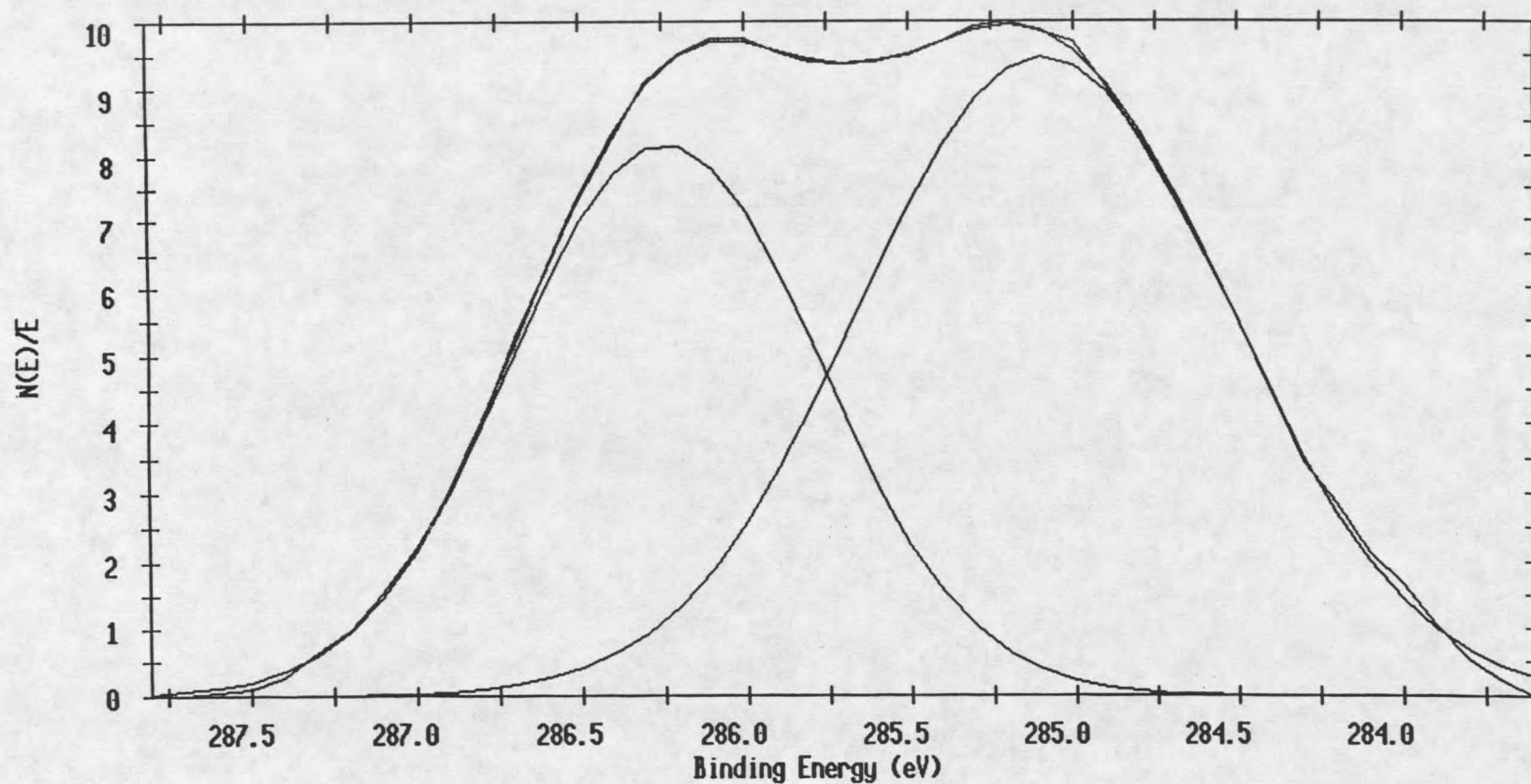


Figure 25: XPS high resolution carbon 1s scan to check for the existence of a proteinaceous conditioning film. The lack of nitrogen, along with the lack of an amide functionality at 288.5 eV shows that a proteinaceous conditioning film is not on the polymer surface.

Whole cells were analyzed to check the elemental composition of the cell surface. A survey scan of this sample is shown in Figure 26. This experiment was repeated three times, and the atomic concentrations are as follows: carbon $62.4 \pm 1.7\%$; oxygen $33.4 \pm 1.9\%$; and nitrogen $4.2 \pm 0.2\%$. This elemental distribution probably reflects the polysaccharides, mannoproteins, and proteins located within the outermost 100 Å of the yeast cell wall.

Poly (vinyl chloride) samples containing "footprints" were also analyzed with XPS to gain an understanding of the chemical nature of the adhesive biomolecules on the surface of the polymer. A survey spectra of this sample is shown in Figure 27.

The existence of sodium, chlorine, and phosphorus, and an increase in oxygen is attributed to the PBS buffer. This can be corrected by subtracting these elements due to the buffer, and normalizing the remaining percentages to 100%. The composition of the "footprint" is shown in Table 4 along with the composition of clean PVC, and whole cells.

Sample	Carbon	Oxygen	Nitrogen	Chlorine
PVC	67.1 ± 0.3	ND	ND	32.9 ± 0.3
WHOLE CELLS	62.4 ± 1.7	33.4 ± 1.9	4.2 ± 0.2	ND
"FOOTPRINTS"	63.2 ± 7.4	30.4 ± 4.9	6.5 ± 2.5	ND

Table 4: Table showing elemental compositions for polymer, whole cells, and "footprints" as determined by XPS.

This table shows a slightly increased level of nitrogen in the "footprint" compared to whole cells. This may mean that proteins, or mannoproteins are the actual adhesins used by the yeast to anchor to the surface. This however, cannot be stated without a reasonable doubt. It does however, show that it is not just a polysaccharide of the organism which

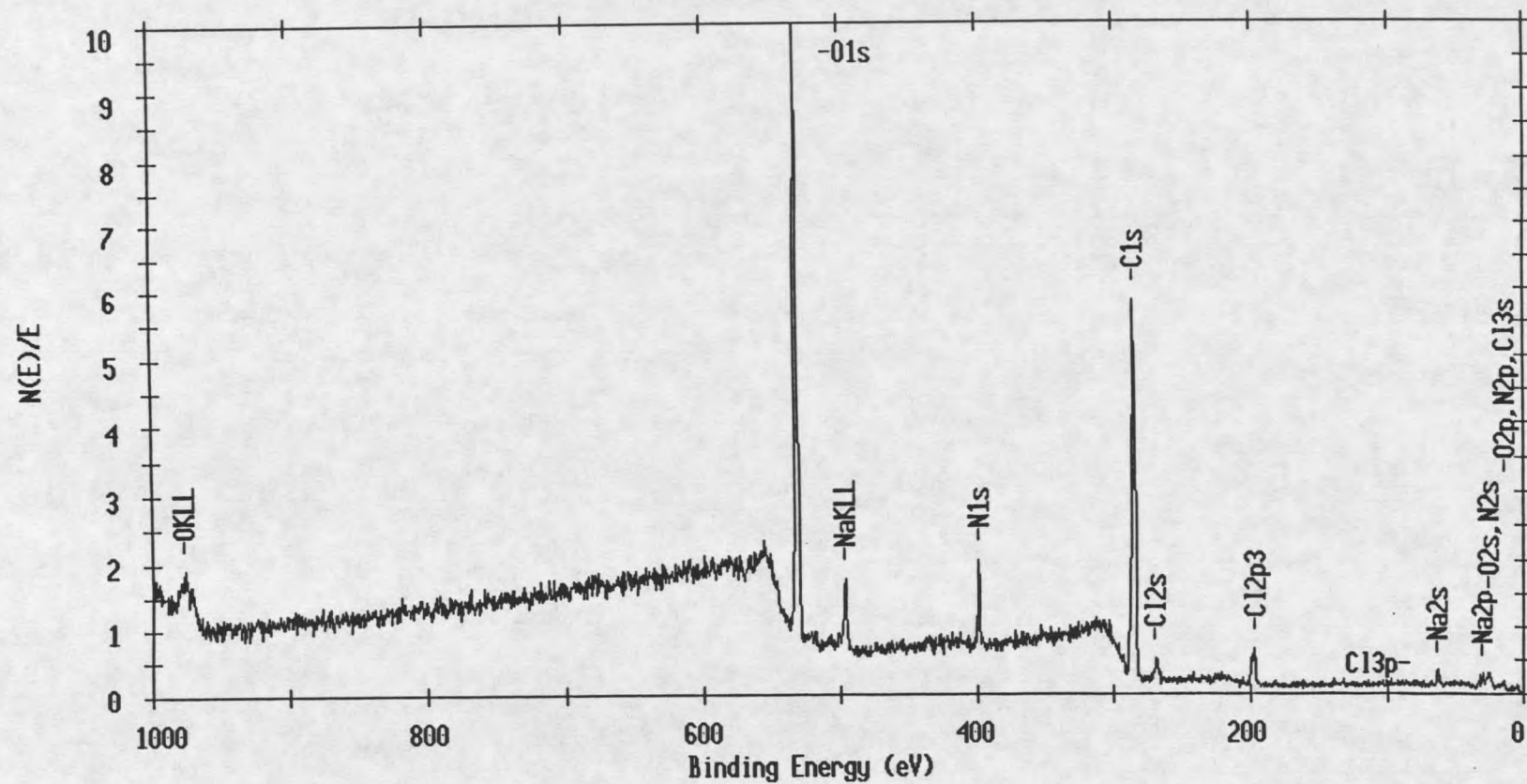


Figure 26: XPS survey spectra of whole *C. albicans* cells. Nitrogen, oxygen, and carbon are present along with chlorine and sodium from the buffer.

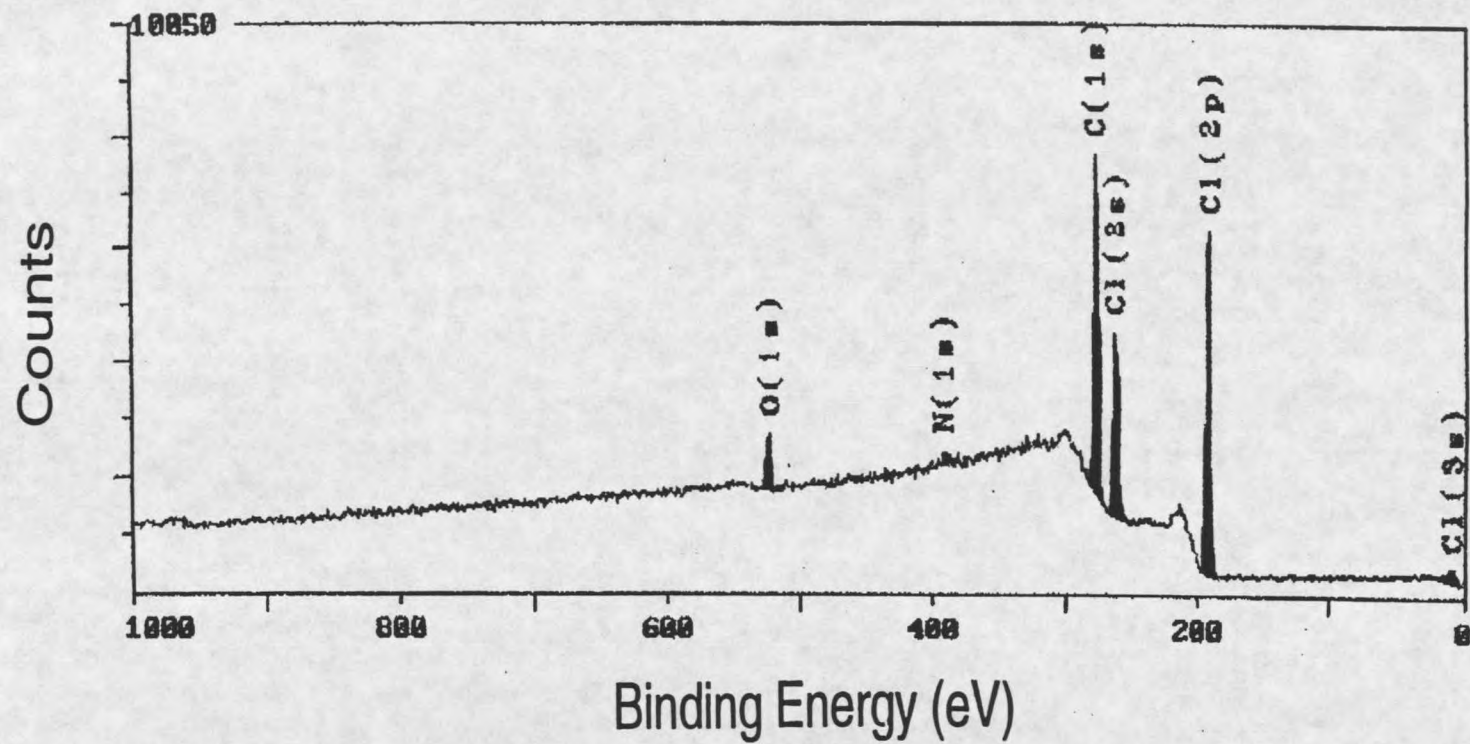


Figure 27: XPS survey scan of poly (vinyl chloride) surface after removal of adherent yeast cells. The sample containing “footprints” now has oxygen and nitrogen present along with the elements of the polymer.

adheres it to the surface. A high resolution Carbon 1s region scan of this sample yields the spectra shown in Figure 28.

This spectra indicates increased levels of C-O and/or C-N functionalities, as well as a peak at 288.5 eV indicative of the existence of amide linkages on the polymer surface. This proves that the nitrogen on the sample comes from either peptide linkages in proteins, or from n-acetylglucosamine, the chemical used as a joint between the carbohydrate and protein portions of glycoproteins. It is therefore apparent that mannoproteins or proteins play a key role in the adherence process as probed by the XPS technique.

4.2.4 Secondary Ion Mass Spectrometry

Clean PVC samples were analyzed by Secondary Ion Mass Spectrometry (SIMS) to study the molecular structure of the polymer surface. The positive ion spectra was dominated by hydrocarbon fragments as shown by Figure 29. This is an expected fragmentation pattern for PVC, with the peaks having the chemical structure of $C_nH_m^+$.⁷¹ The negative ion spectra, Figure 30, was totally dominated by peaks at 35 and 37 amu indicative of the two isotopes of chlorine. Very few other negative ion peaks were observed.

SIMS analysis of "footprint" samples yielded interesting results on the molecular structure of the "footprints". This spectra was analyzed by subtracting it from the PVC background spectra with a normalization to total counts method used. The contribution of the buffer was then subtracted from this spectra, and a resulting spectra indicative of the "footprint" material was obtained. Figures 31 and 32 show the resulting spectra from 0-

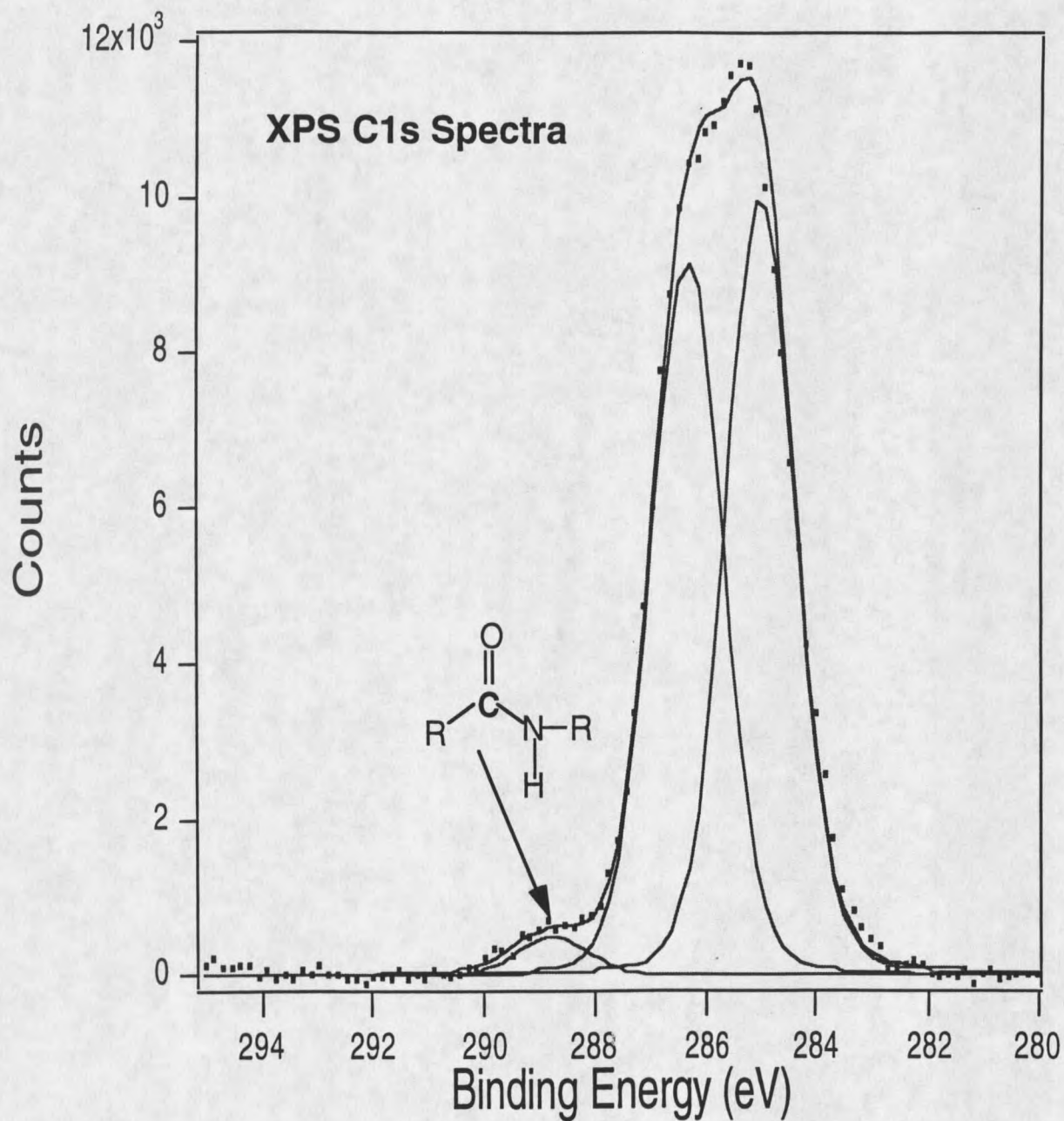


Figure 28: XPS high resolution carbon 1s region. Note the existence of a peak at 288.5 eV indicative of an amide linkage from either a protein or mannoprotein.

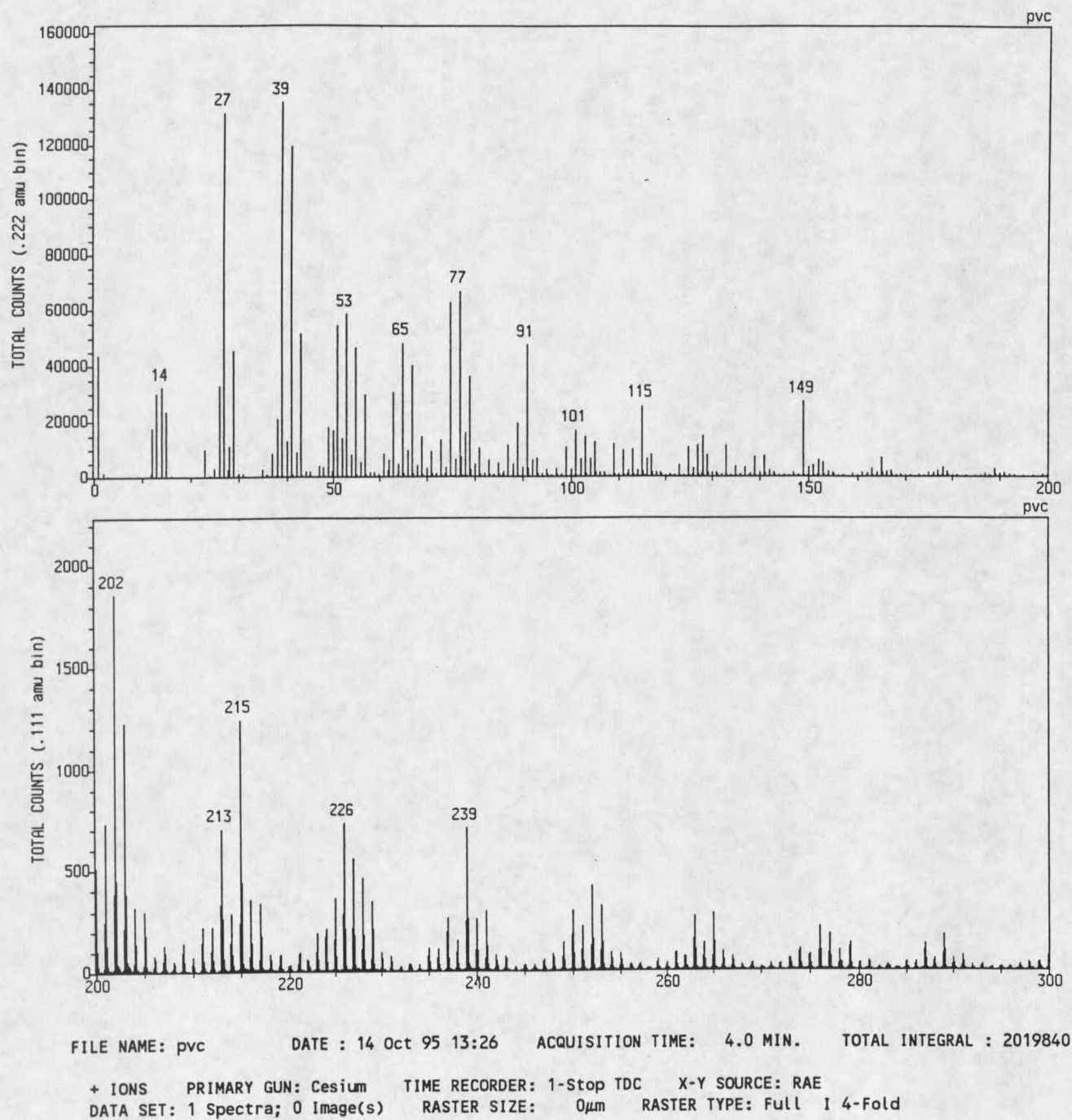
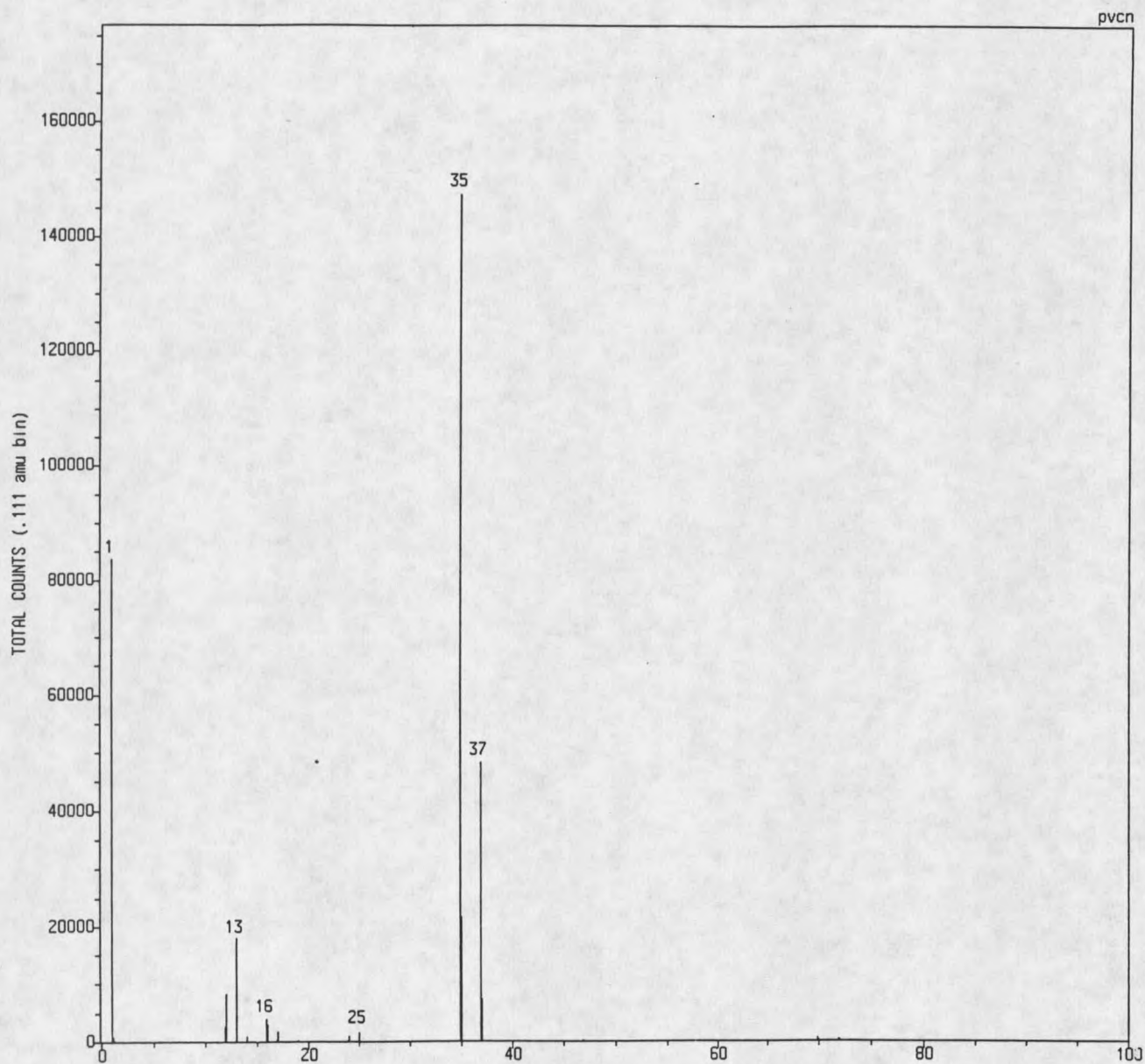


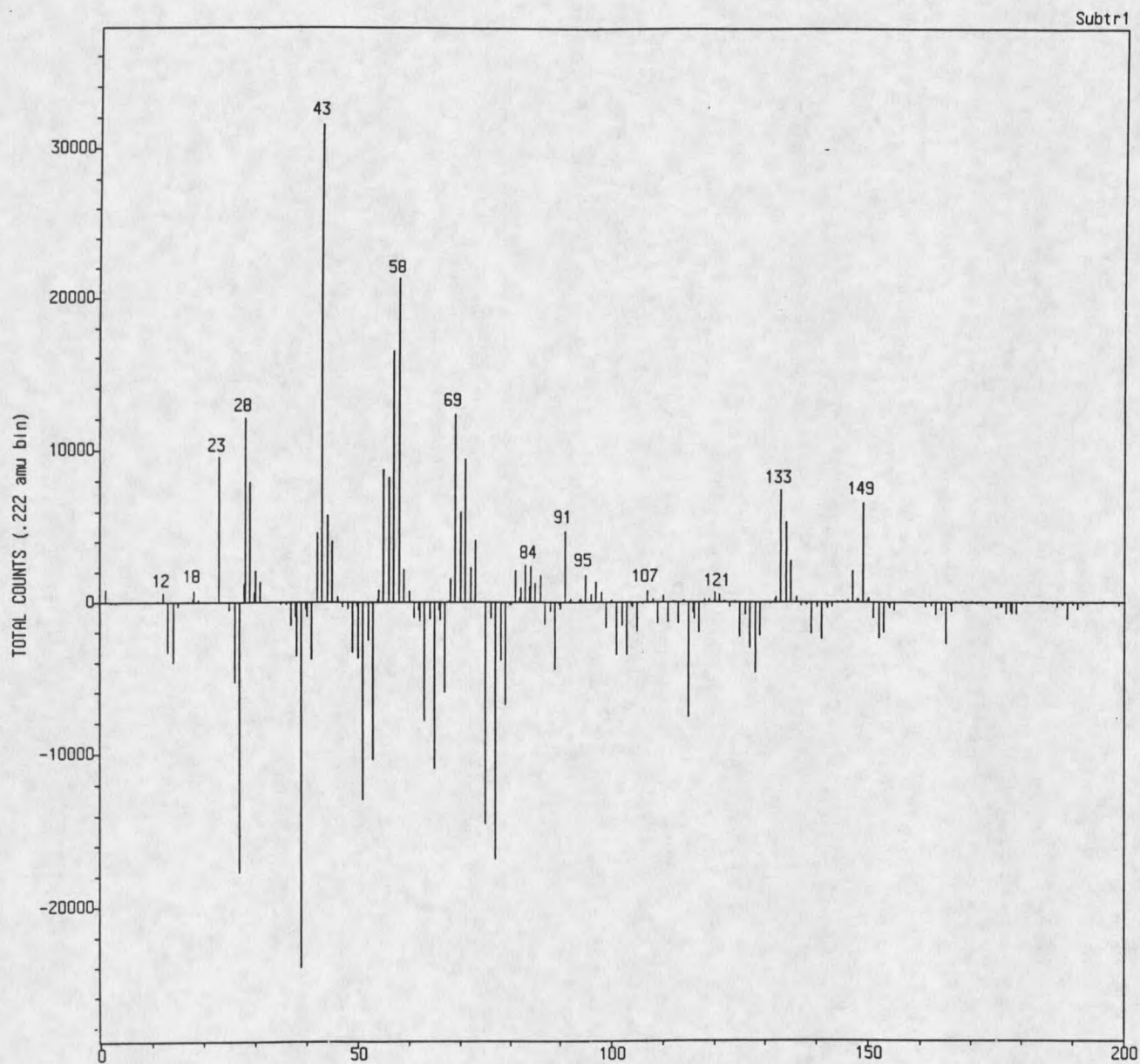
Figure 29: SIMS positive ion spectra for poly(vinyl chloride).



FILE NAME: pvcn DATE : 18 May 95 16:40 ACQUISITION TIME: 2.1 MIN. TOTAL INTEGRAL : 359424

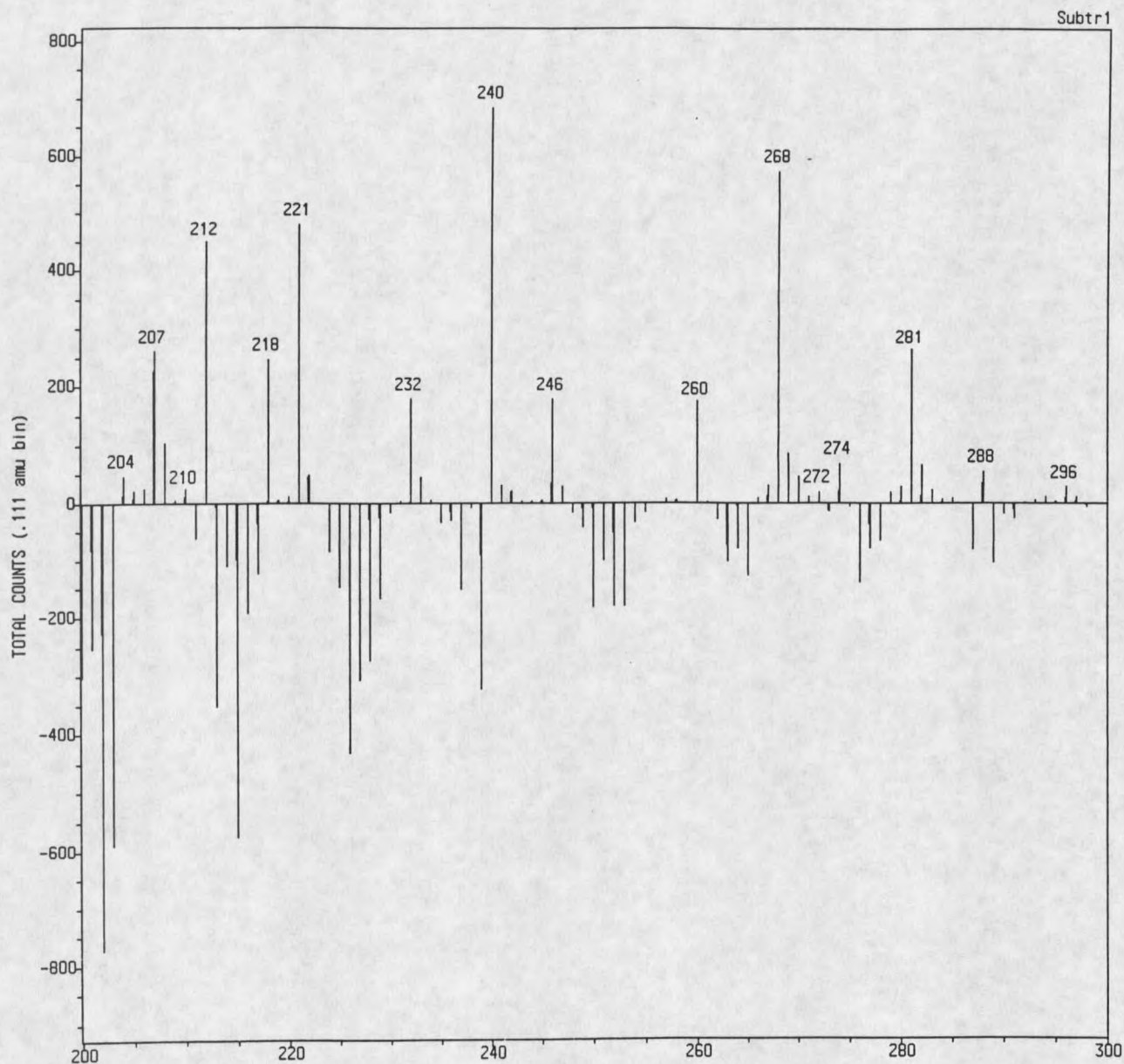
- IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: Raster
DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 0 μ m RASTER TYPE: Full I 4-Fold

Figure 30: SIMS negative ion spectra of poly(vinyl chloride).



FILE NAME: Subtr1 DATE : 23 Oct 95 19:32 ACQUISITION TIME: 0 SEC. TOTAL INTEGRAL : -1890
Peak Area Diff. (Subtr4-GlBuf) over 1000.0 mamu range; Norm: Total Cts; Ratio=-0.003

Figure 31: SIMS positive ion spectra from 0-200 amu of the “footprint” after subtraction of the poly(vinyl chloride) and buffer contributions.



FILE NAME: Subtr1 DATE : 23 Oct 95 19:32 ACQUISITION TIME: 0 SEC. TOTAL INTEGRAL : -1890
Peak Area Diff. (Subtr4-GlBuf) over 1000.0 mamu range; Norm: Total Cts; Ratio=-0.003

Figure 32: SIMS positive ion spectra from 200-300 amu of the “footprint” after subtraction of the contributions from poly(vinyl chloride) and the buffer.

200 amu and 200-300 amu respectively. Figure 33 shows the "footprint" spectra before any manipulation.

In the 0-200 amu range, the heightened areas seem to be mainly due to hydrocarbon fragments such as peaks at 43, 55, 69. These are most likely due to fragmentation of polysaccharides present in the "footprint".⁷² The region of 200-300 amu as shown in Figure 32, indicates the existence of many even numbered ion peaks. Even numbered peaks are usually indicative of nitrogen containing compounds such as proteins, or glycoproteins. A pattern can be seen in this spectra. At mass 288 there is a peak, followed by peaks at 14 amu less throughout the 200 region. This could be indicative of a number of different proteins present. Peaks which are below the centerline, are indicative of species present in the PVC or buffer, while not present in the "footprint". The negative ion subtracted spectra, obtained in the same manner is shown in Figure 34. This spectra, however yields little information as the subtracted spectra has few peaks and indicates little difference between the "footprint" spectra and the background PVC. The unedited "footprint" spectra is shown in Figure 35. The specific structure of the molecules of the "footprint" are not know, however it appears that proteins, polysaccharides, and/or mannoproteins are present in the "footprint".

A study was also conducted to study the fragmentation pattern of purified *Candida albicans* cell wall antigens. The fragmentation pattern was then compared to previous work done by Yong He using a quadrupole mass analyzer.⁷² The positive ion fragmentation patterns (shown in Figure 36) compare well with those obtained by Yong He (Figures 37, 38, 39). The spectra is dominated by hydrocarbon fragments, and the only differences appear to be slight ones in peak intensities. This is most likely due to differences in transmission

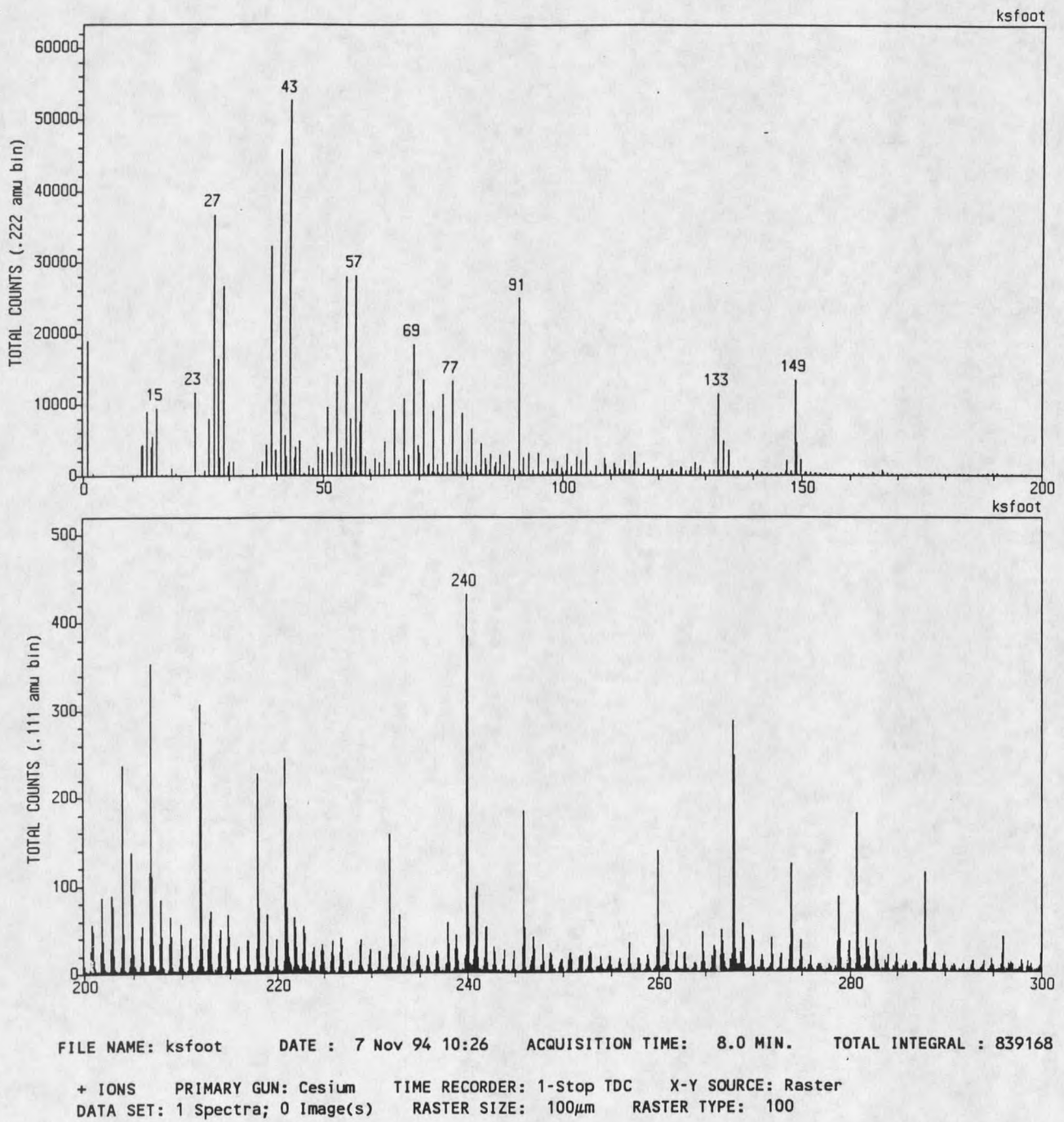
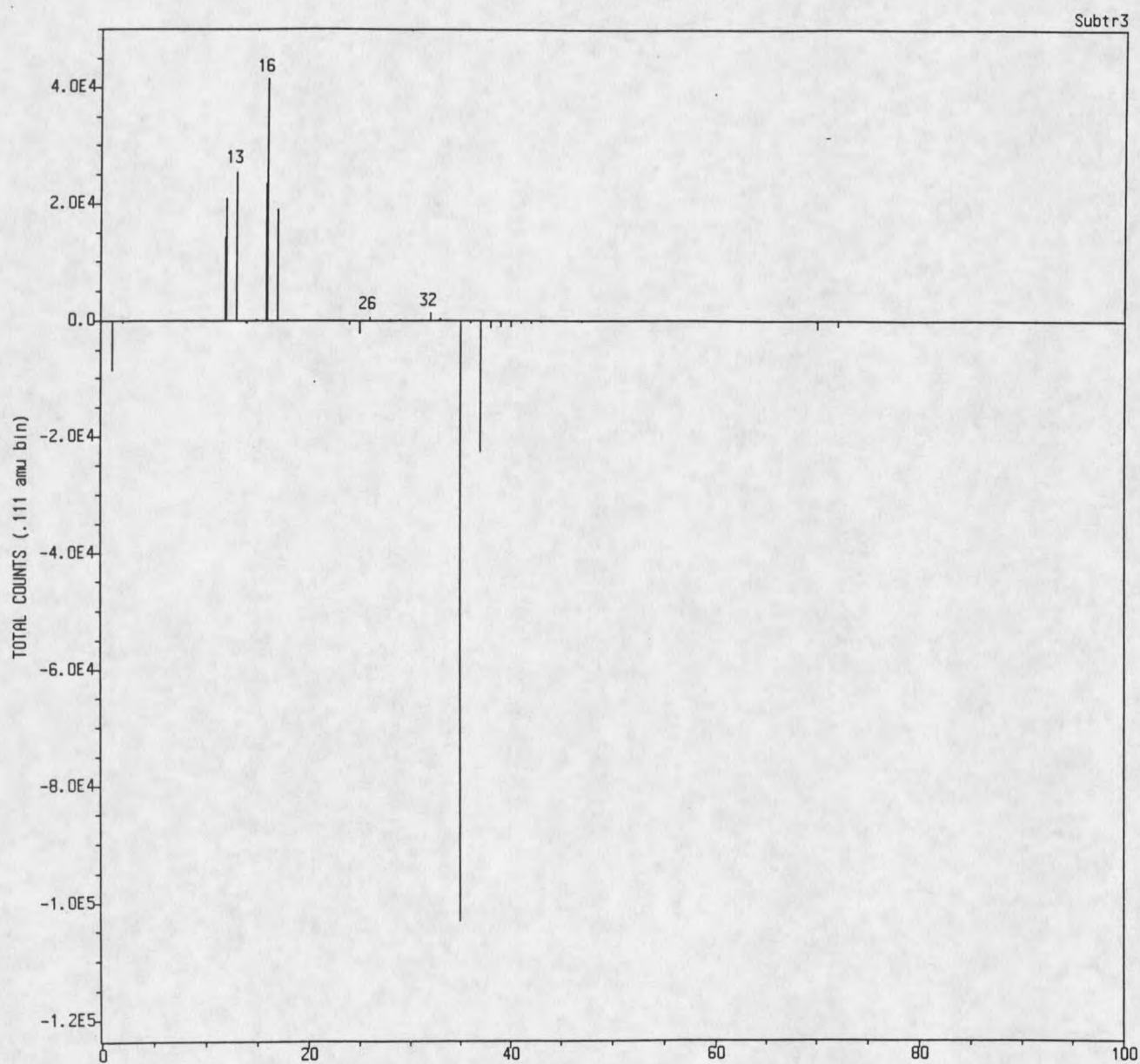
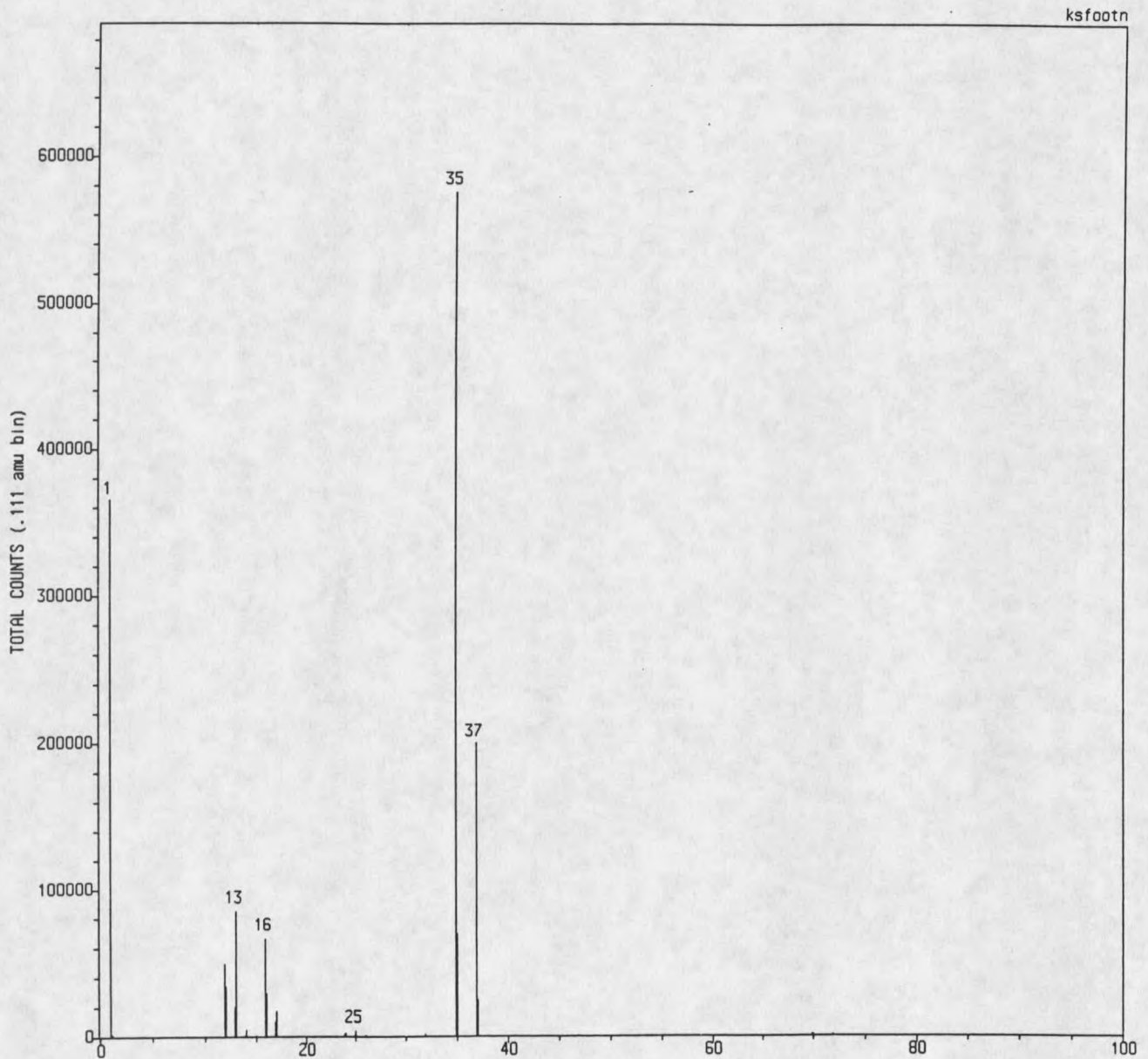


Figure 33: SIMS positive ion spectra of "footprint" sample before any manipulation.



FILE NAME: Subtr3 DATE : 30 Oct 95 8:43 ACQUISITION TIME: 0 SEC. TOTAL INTEGRAL : -171
Peak Area Diff. (Subtr4-GlBuf-) over 1000.0 mamu range; Norm: Total Cts; Ratio=1E-3

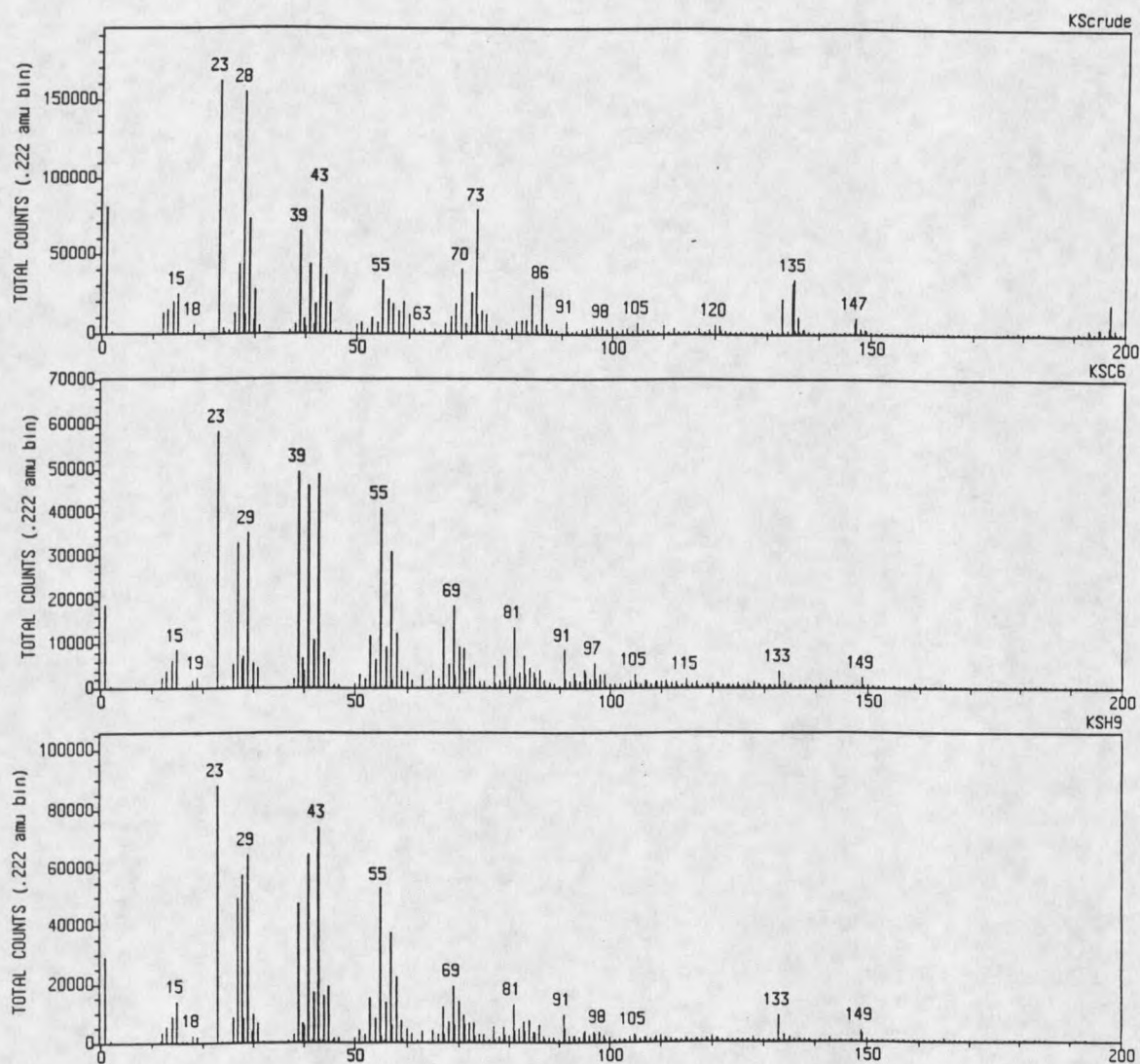
Figure 34: SIMS negative ion spectra of "footprint" after subtraction of background contributions from poly(vinyl chloride) and the buffer.



FILE NAME: ksfootn DATE : 7 Nov 94 11:11 ACQUISITION TIME: 8.0 MIN. TOTAL INTEGRAL : 1593344

- IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: Raster
DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 0 μ m RASTER TYPE: 0

Figure 35: SIMS negative ion spectra of "footprint" sample before any data manipulation.



FILE NAME: KScrude DATE : 10 Oct 95 13:03 ACQUISITION TIME: 5.0 MIN. TOTAL INTEGRAL : 2000896
 Crude antigen; positive ions
 + IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: RAE
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 65 μ m RASTER TYPE: Full I 4-Fold

FILE NAME: KSC6 DATE : 10 Oct 95 13:33 ACQUISITION TIME: 3.0 MIN. TOTAL INTEGRAL : 869888
 Antigen C6; positive ions
 + IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: RAE
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 65 μ m RASTER TYPE: Full I 4-Fold

FILE NAME: KSH9 DATE : 10 Oct 95 13:43 ACQUISITION TIME: 3.8 MIN. TOTAL INTEGRAL : 1212928
 Antigen H9; positive ions
 + IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: RAE
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 65 μ m RASTER TYPE: Full I 4-Fold

Figure 36: TOF-SIMS positive ion spectra of a) crude cell wall extract b) purified cell wall antigen C6 and c) purified cell wall antigen H9.

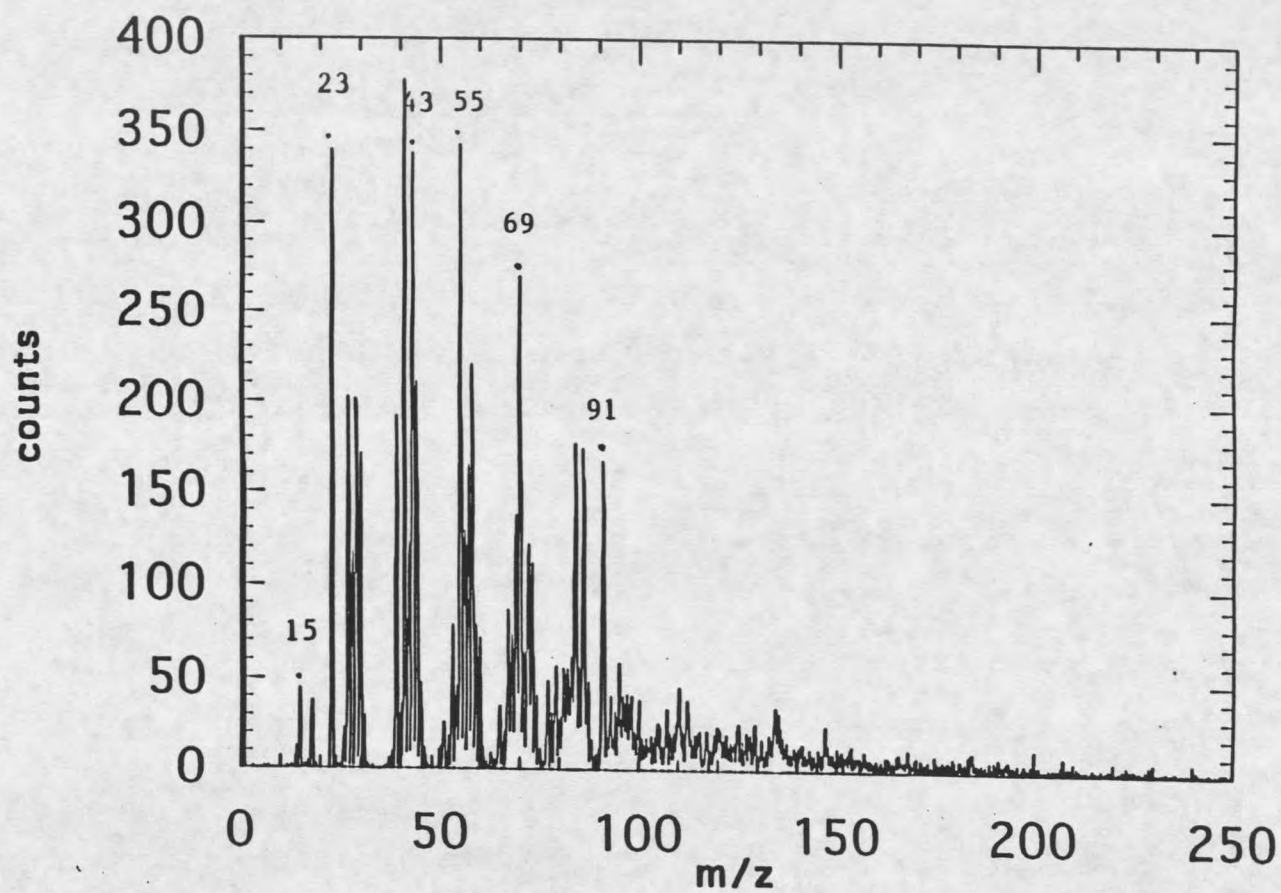


Figure 37: Quadrupole SIMS positive ion spectra of crude *C. albicans* cell wall extract. Spectra obtained by Yong He.

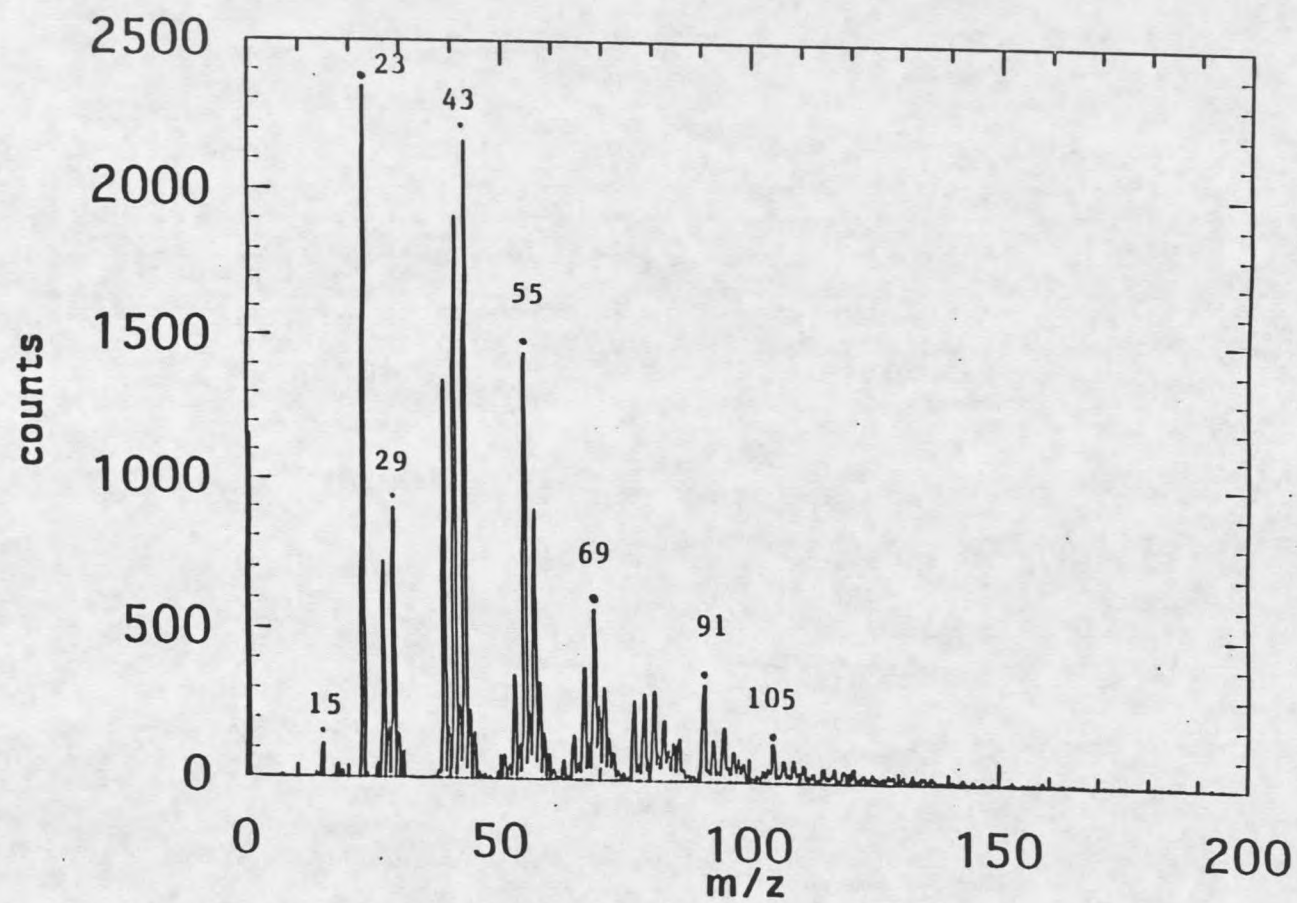


Figure 38: Quadrupole SIMS positive ion spectra of purified *C. albicans* cell wall antigen C6. Spectra obtained by Yong He.

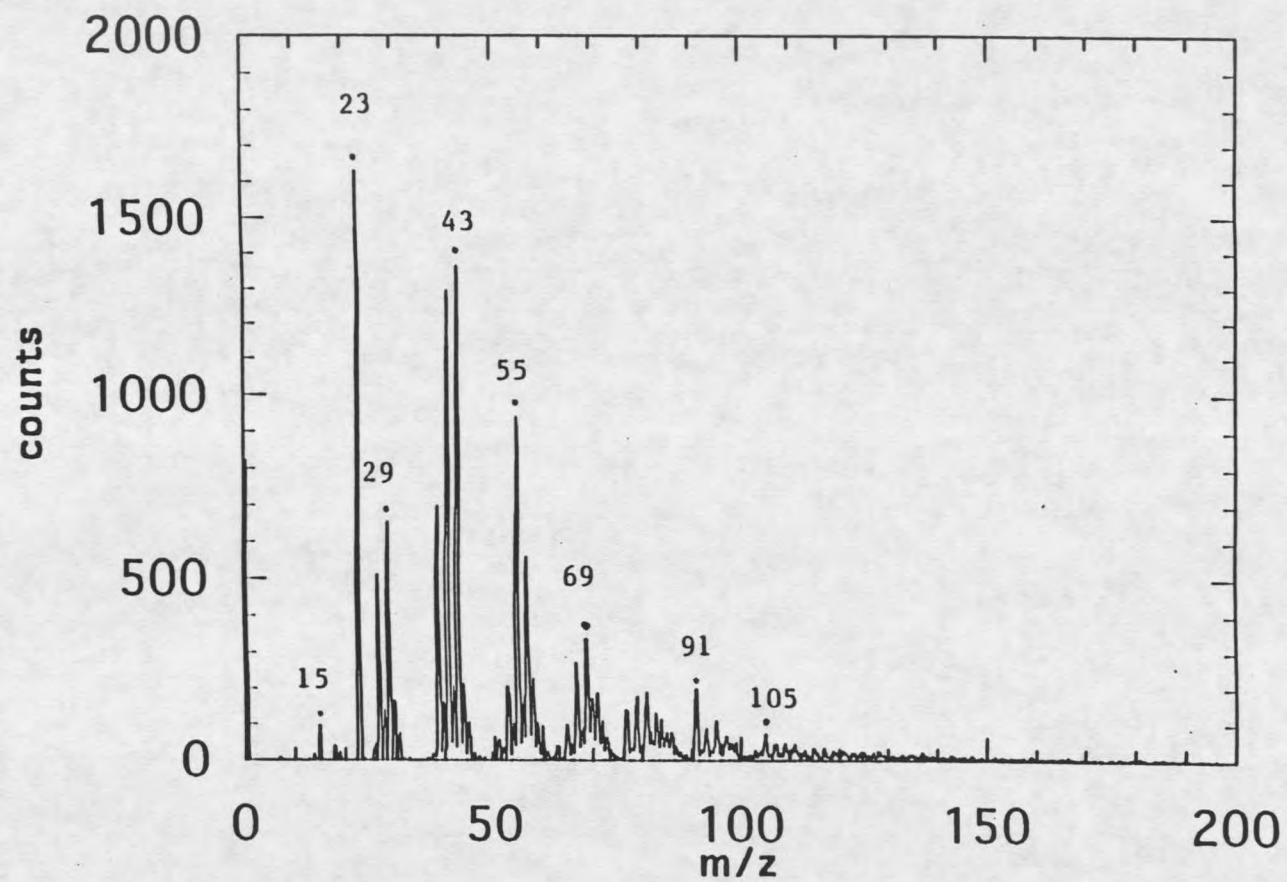


Figure 39: Quadrupole SIMS positive ion spectra of purified *C. albicans* cell wall antigen H9. Spectra obtained by Yong He.

between the two mass analyzer types. The negative ion spectra, Figure 40, however, drastically differs from those obtained by He, Figures 41, 42, and 43. The TOF-SIMS spectra is not dominated by peaks at 26 and 42 amu, characteristic of protein fragmentation. The quadrupole SIMS results are nearly dominated by the 26 and 42 amu peak in all cases. The fragmentation patterns should be similar, so this shows that the transmission and detection results are slightly different for the quadrupole SIMS used by He, and the TOF-SIMS instrument used for these experiments.

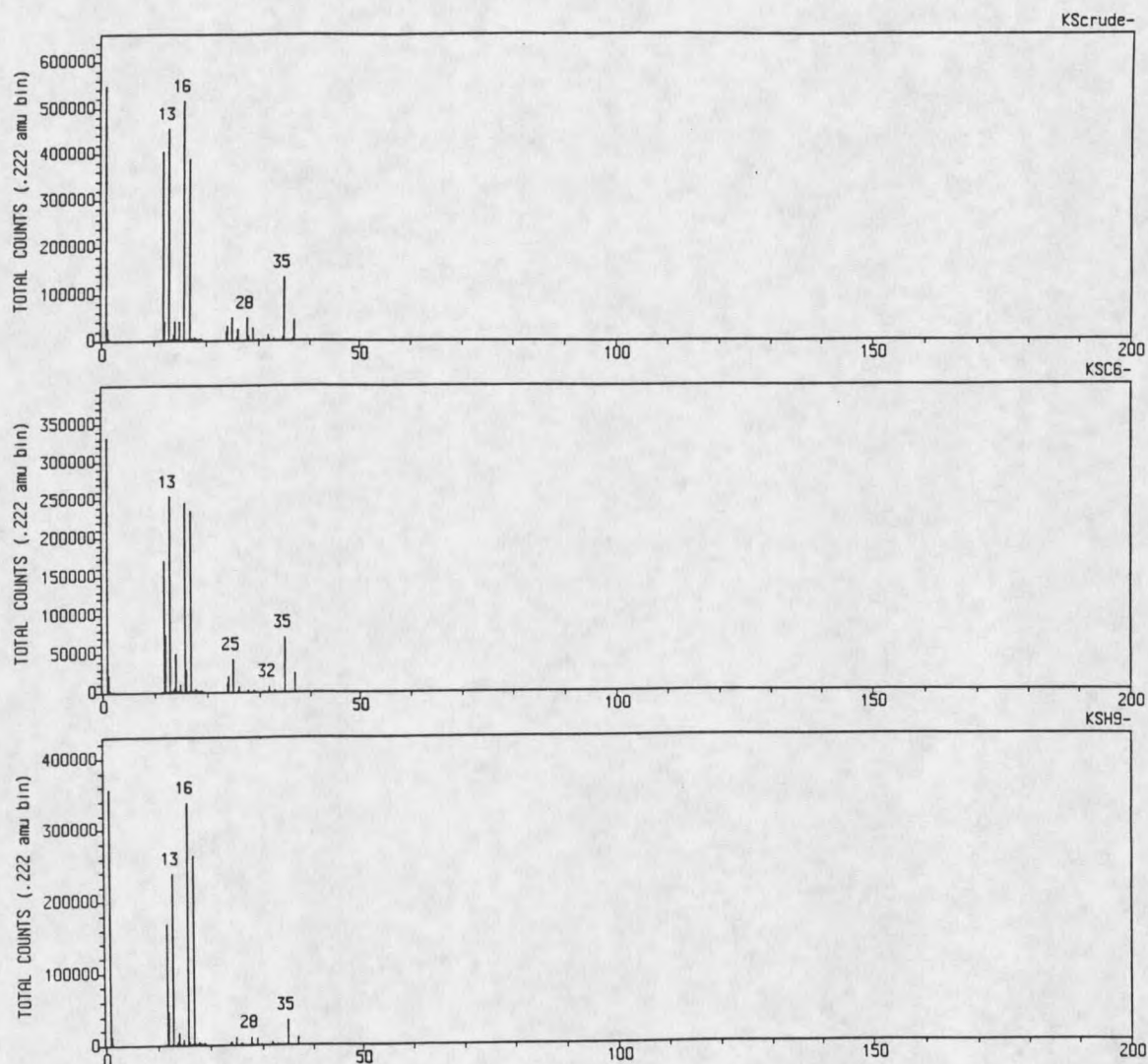
4.2.5 Contact Angle

Contact angle results yielded good information about the surface energetics of the PVC films. Drop measurements were collected and correlated to contact angles using equations 2 and 3. The contact angles for the liquids used are listed in Table 5.

Contact angle data was then plotted as surface tension of the liquid versus cosine of the contact angle. This is a typical Zisman type plot with the intercept being the critical surface tension of the sample. This plot is shown in Figure 44.

Liquid	Surface Tension (dynes/cm)	Contact Angle (°)	Standard Deviation	
Water	72.4	99.7 ^{59.9}	1.4	718.24
Glycerol	64.8	75.9 ^{80.6}	2.7	37.21
Diiodomethane	49.0	28.0 ^{92.3}	1.3	31.56
Ethylene Glycol	47.9	26.2 ^{90.9}	1.8	33.64
Propylene Carbonate	41.8	0.0 ^{83.6}	0.0	9.61

Table 5: Table showing contact angle liquids used, contact angles, and standard deviations.



FILE NAME: KScrude- DATE : 10 Oct 95 13:23 ACQUISITION TIME: 5.1 MIN. TOTAL INTEGRAL : 3012096
 Crude Antigen; negative ions
 - IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: RAE
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 65 μ m RASTER TYPE: Full I 4-Fold

FILE NAME: KSC6- DATE : 10 Oct 95 13:38 ACQUISITION TIME: 3.0 MIN. TOTAL INTEGRAL : 1710592
 Antigen C6; negative ions
 - IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: RAE
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 65 μ m RASTER TYPE: Full I 4-Fold

FILE NAME: KSH9- DATE : 10 Oct 95 13:50 ACQUISITION TIME: 3.0 MIN. TOTAL INTEGRAL : 1626112
 Antigen H9; negative ions
 - IONS PRIMARY GUN: Cesium TIME RECORDER: 1-Stop TDC X-Y SOURCE: RAE
 DATA SET: 1 Spectra; 0 Image(s) RASTER SIZE: 65 μ m RASTER TYPE: Full I 4-Fold

Figure 40: TOF-SIMS negative ion spectra of a) crude cell wall extract b) purified cell wall antigen C6 and c) purified cell wall antigen H9.

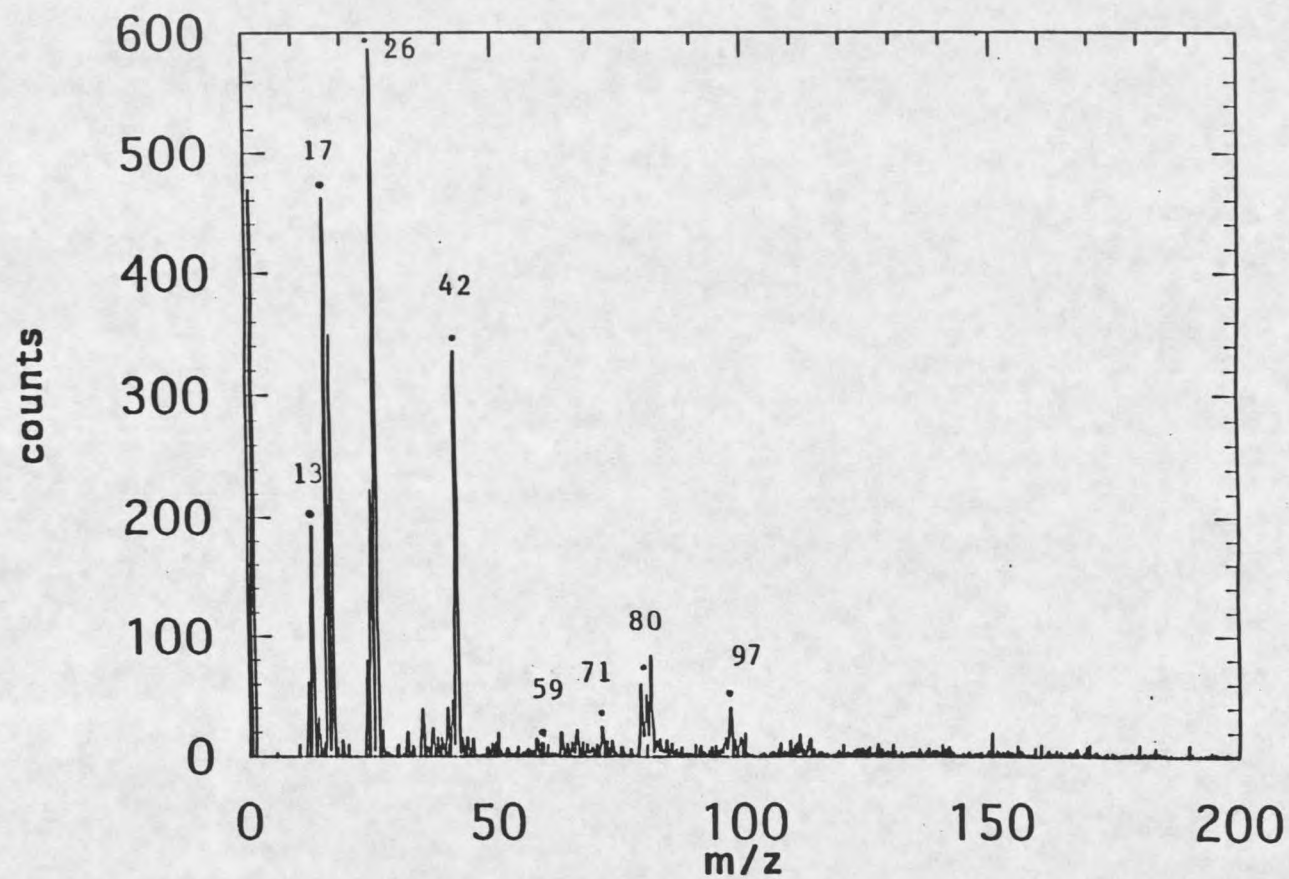


Figure 41: Quadrupole SIMS negative ion spectra of crude *C. albicans* cell wall extract. Spectra obtained by Yong He.

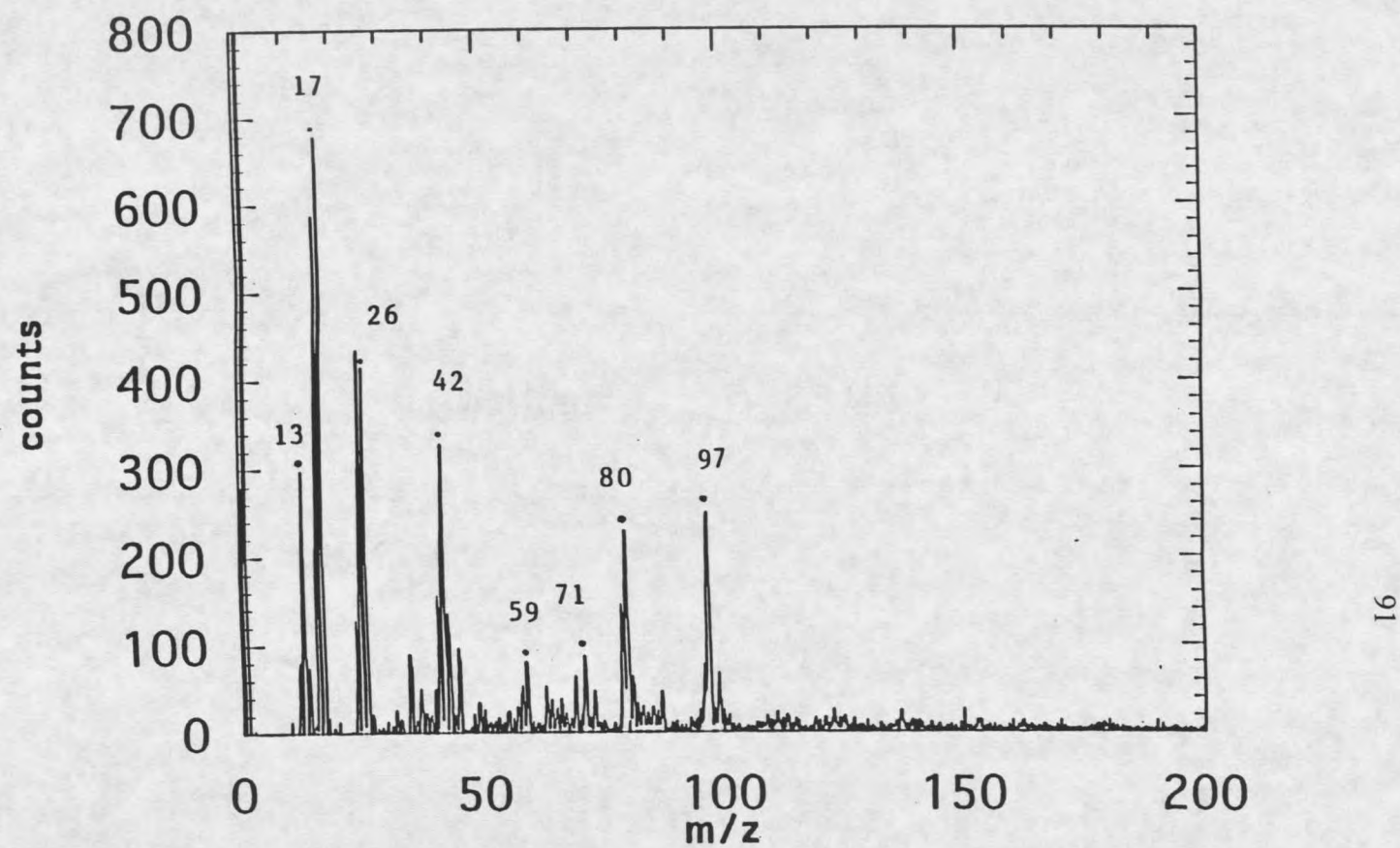


Figure 42: Quadrupole SIMS negative ion spectra of purified *C. albicans* cell wall antigen C6. Spectra obtained by Yong He.

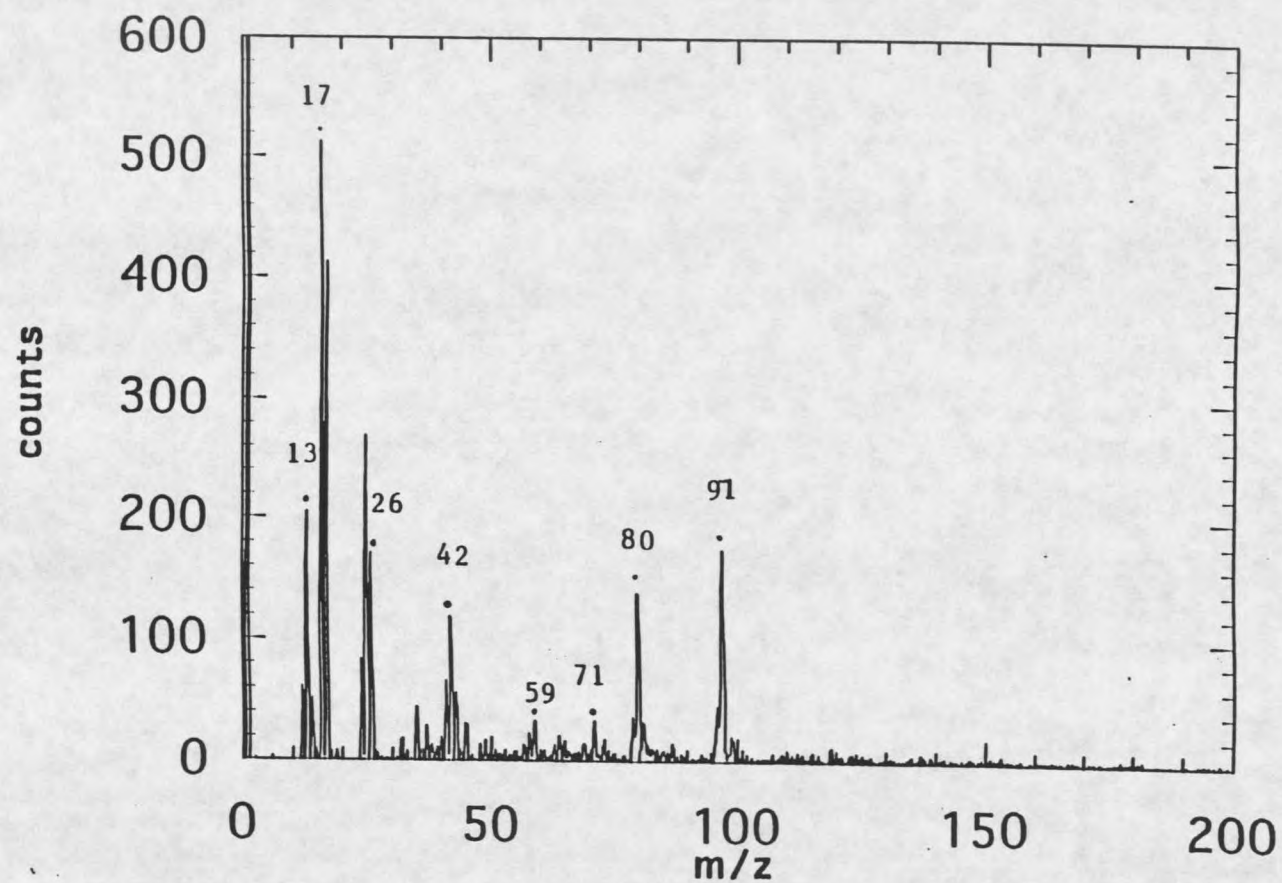


Figure 43: Quadrupole SIMS negative ion spectra of purified *C. albicans* cell wall antigen H9. Spectra obtained by Yong He.

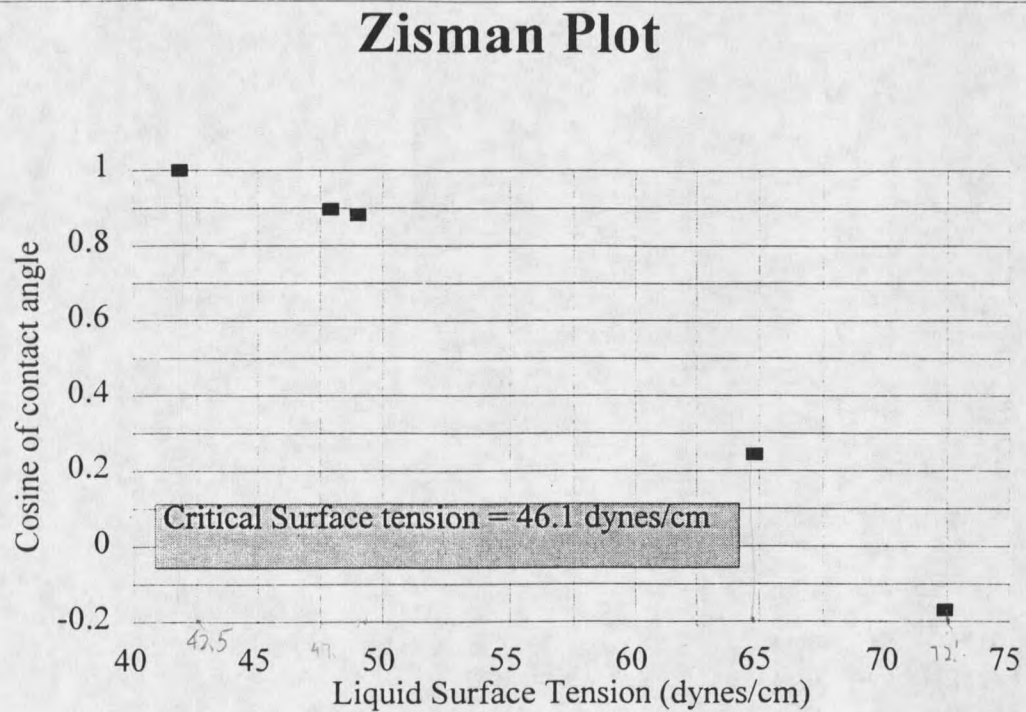


Figure 44: Zisman plot of contact angle results. The intercept is indicative of the critical surface tension of the sample.

The expected literature value for the critical surface tension of poly(vinyl chloride) is 39 dynes/cm. This technique therefore yielded a critical surface tension of the PVC samples slightly higher than the expected value (46.1 dynes/cm). The deviation may be due to impurity in the liquids, or the fact that this is a spun-cast, plasticizer-free, high molecular weight PVC sample. However, this shows that the PVC films created have defined surface energetics close to that given in the literature.

4.2.6 Fourier Transform Infrared Spectroscopy

The flow cell designed for this project will now be described in detail. As stated before, the design limitations included the following: it must incorporate a rectangular geometry Internal Reflection Element (IRE) made of Germanium, it must allow in-situ observation by a microscope, and the IRE must be removable.

To accomplish this task, a HORIZONTM mirror assembly was acquired from Harrick Scientific. This mirror assembly used two flat optical mirrors and a concave optical mirror to focus the beam into the IRE and a concave optical mirror and two flat optical mirrors to focus the beam exiting the IRE into the detector. The key design parameter of this assembly was the location of the beam focussing. Since the assembly was purchased without a top, a top had to be designed to position the IRE crystal to line up with the FTIR beam. This mirror assembly was then used in conjunction with a Nicolet 740 FTIR unit.

To accomplish this task, an aluminum plate was created to fit tightly into two pins located on the top of the HORIZONTM assembly. Two 1 inch circles were cut into the aluminum and connected by a 2.8 mm deep groove, 50 mm long, and 20 mm wide. This

groove was the position of where the IRE crystal would be located in the flow cell.

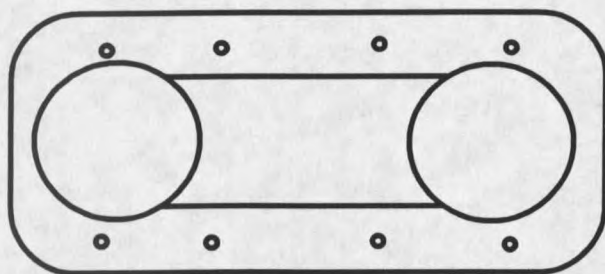
With the base plate in position, a top and flow channel had to be engineered to finish the flow cell design. A 1/32 inch silicon gasket was cut into a 20 mm x 48 mm rectangle. The gasket was created by removing all but the outermost 3 mm of the gasket. The silicon gasket was used as a spacer to correctly position the IRE, as well as a shock absorber for the crystal as the top was placed onto it. The silicon gasket was covered with a thin piece of aluminum, 20 mm x 48 mm. This was done to mask the highly infrared absorbing silicon from the IRE. A 50 mm x 20 mm x 2 mm thick Germanium IRE with a 45° trapezoidal geometry scheme was selected as the IRE of choice. Germanium has a high refractive index which made it the chosen material for the IRE.

The IRE was placed onto the silicon/aluminum gasket material. The flow channel was created by placing a 20 mm x 48 mm viton rubber gasket onto the crystal. The inside of the viton gasket was removed leaving a thickness of 2 mm all around the edges of the gasket. The gasket thickness chosen was 1/32 inch, corresponding closely to the thickness of the teflon flow cell. This was done because at this thickness, microscope objectives with a feasible working distance were readily available.

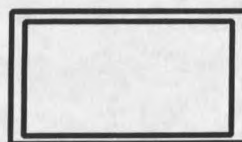
The top plate of the flow cell was constructed of 316 stainless steel. This material was chosen because it is chemically resistant to base bath making it readily cleanable. Inlet and outlet ports for the top were constructed from 1/32 inch I.D., 1/16 inch O.D., 316 stainless steel tubing, gold-soldered into holes in the top plate. Gold solder was chosen because of its resistance to chemical attack. The tubing was fit with Swagelok[®] brand fittings to allow easy connections to be made with teflon tubing. A rectangle with dimensions of 16

mm by 34.5 mm was recessed 0.2 mm into the bottom of the top plate to allow insertion of a specially prepared #1 thickness glass cover slip. This glass piece was then placed into the groove with the aid of epoxy resin, creating the top of the flow cell as well as a viewing port for the microscopic observation portion of the design requirement. The top possessed 45° beveled edges to allow easier insertion of the microscope objective. A diagram of the flow cell is shown in Figures 45 and 46. A polycarbonate box was then built around the mirror assembly to allow the system to be purged with dry air.

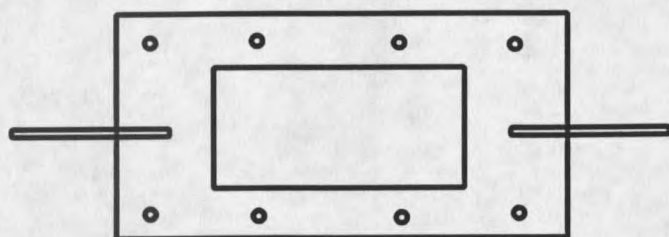
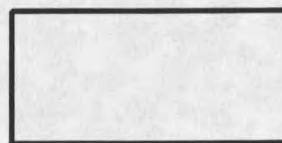
The flow cell worked both from a microscopic observation point of view, and from a chemical analysis point of view. Preliminary data on the adhesion of *C. albicans* to the Germanium IRE was investigated by others. The flow cell was found to have high infrared throughput, minimal leakage, and good microscopic observation potential. It was therefore deemed a successful design, and no studies for this thesis were conducted with it.



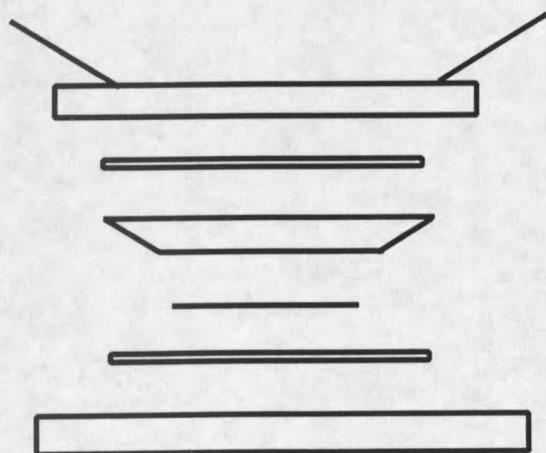
Al Base Plate



Gasket

Flow Cell Top
w/ Viewing Port

Ge IRE

Figure 45: Top view diagram of the FTIR/ATR flow cell constructed.

Top Plate

Viton Gasket

Ge IRE

Al Foil

Si Gasket

Al Base Plate

Figure 46: Side view of the FTIR/ATR flow cell as assembled.

CHAPTER 5

SUMMARY

This thesis presents results of the study of the initial attachment events of *Candida albicans* to poly(vinyl chloride) in a dynamic system. Culture variables, as well as process variables, were found to have a significant impact on yeast cell adherence. Yeast cells grown in glucose-based medium were found to adhere in greater numbers to the polymer than galactose-grown cells. Shear rate was a critical factor in the adherence process. As shear rate increased, a significant decrease in cell adhesion was noticed. However, shear rate of attachment did not affect the tenacity with which the cells were bound to the surface. Shear rate effects were more dramatic for glucose-grown cells than for galactose-grown cells. Growth parameters varied depending on carbohydrate source. Although total cell numbers were nearly identical after 24 hours of growth, the number of cells per cluster was dramatically higher for glucose grown cells. Glucose-grown cells were also found to be roughly 1 μm larger in diameter than galactose grown cells. Adherence kinetics were linear for both carbohydrate sources except at shear rates greater than 100 sec^{-1} where the linearity was no longer observed as a doubling was not seen between 15 and 30 minutes of adherence time. The kinetic curve became non-linear at 46 sec^{-1} shear rate after only 30 minutes of adherence time. This may be caused by the increased surface coverage of adherent yeast and decreased availability of bare polymer areas.

Two theories have been proposed for the understanding of this process. The first is based upon a free energy driving force. As the cell adheres to the surface, it must displace

a small amount of water bound to the polymer surface. Once initially attached, it very quickly displaces more bound water, allowing it to tenaciously bind to the surface within a few seconds or minutes. The free energy driving force is dependent on the structure of the cell wall, which varies with carbohydrate source. The second is based upon the fact that a mechanical linkage is formed between the surface of the polymer, and the yeast cell surface. The mechanism of linkage is not dependent on carbohydrate concentration, but is dependent on carbohydrate source. It therefore may be a structural difference in the cell wall moieties that promote adhesion. The fibrillar layer, known to be enhanced by growth in galactose, may produce adhesins more closely packed together allowing multiple sites to adhere at the same time. This multiple linkage would allow the cell a better chance of adhering in a high shear field. However, these attachments may not be easily formed. The attachments are strong, as evidenced by increased adherence in high shear fields, but are not easily formed as evidenced by lower adherence rates of galactose-grown cells as compared to glucose-grown cells. Glucose adhesins may be longer and less dense, allowing the cell to be able to link to the surface from a greater distance, thereby explaining the increased adherence seen with glucose grown cells at the lower shear rates. The glucose-grown cell mechanism may easily attach to the surface, however, these bonds may not be as strong as the bonds created by galactose-grown cells.

Adhesive biomolecules were found on the surface after removal of adherent yeast cells. These biomolecules were labeled "footprints". The "footprints" had a distinct morphology and were roughly equal in size to the yeast cell. Atomic Force Microscopy and Scanning Electron Microscopy techniques yielded similar images of the "footprint".

"Footprints" were roughly 4-5 μm in diameter, and consisted of a large globule of residue, surrounded by smaller patches of residue. This supports the structural attachment effect, where each of the globules represents an adhesin contact. The large globule may be the initial attachment site of the cell. It was also found that not all adherent yeast cells left "footprints" on the polymer surface after the cell was removed. X-Ray Photoelectron Spectroscopy analysis yielded a chemical composition of the "footprints" that was comparable to the atomic percentages of the cell wall. The composition of the "footprints" was $63.2 \pm 7.4\%$ carbon, $30.4 \pm 4.9\%$ oxygen, and $6.5 \pm 2.5\%$ nitrogen as compared with a composition of the cell wall of $62.4 \pm 1.7\%$ carbon, $33.4 \pm 1.9\%$ oxygen, and $4.2 \pm 0.2\%$ nitrogen. A peak at 288.5 eV in the XPS spectra revealed the presence of peptide linkages in the chemical residues of the "footprint". These linkages could come from either proteins or glycoproteins present in the cell wall. This however, indicates that the adhesin is not strictly an adhesive polysaccharide. XPS results also demonstrated the lack of a proteinaceous conditioning film on the polymer surface after submersion into an aqueous environment. This shows that the interactions studied by this thesis are cell-polymer interactions, and not specific cell-protein interactions. Secondary Ion Mass Spectrometry yielded mass peaks indicative of proteins and polysaccharides in the positive ion spectra. The negative ion spectra produced few meaningful results. Contact angle experiments yielded the critical surface tension of the PVC films to be near 45 dynes/cm, slightly higher than reported in the literature. A FTIR/ATR flow cell to do simultaneous microscopic observation/chemical analysis has been described in detail and has been shown by others to be operational.

Further studies in this area should focus on changing the cell surface and looking for differences in the adhesive biomolecules on the surface after yeast cell removal. A non-trivial experiment would be to attempt to fractionate the cell wall into its various components and look at the mass fragmentation pattern of these chemicals. A comparison of the various chemicals to the adhesive "footprint" could then be studied to attempt to isolate the specific moieties of the "footprint". Also, different polymer surface chemistries should be investigated to see if specific functionalities on the polymer surface inhibit or promote adhesion. Growth conditions should also be varied in an attempt to correlate cell surface characteristics to adhesion results. This may provide information relating cell structure and functionality to increased/decreased cell adherence. Further chemical analysis of the "footprints" should be completed using the FTIR/ATR flow cell constructed as well as additional SIMS analysis.

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APPENDIX
TEFLON FLOW CELL DATA

ADHERENT CELL COUNTS AFTER 30 MINUTES (GALACTOSE SOURCE)

Shear Rate (Inv. Sec)	1% Galactose			3% Galactose			5% Galactose			9% Galactose		
	Cells per mm2	Std. Dev	n	Cells per mm2	Std. Dev	n	Cells per mm2	Std. Dev	n	Cells per mm2	Std. Dev	n
28				1770	330	12	1960	0.16 320	12			
46	1980	360	14	2100	230	13	2290	0.12 280	13	1920	290	12
71				1590	190	12	1620	0.18 290	12			
120				1160	240	12	1190	0.22 260	15			
350							490	0.18 90	6			

0.12 ~ 0.22

ADHERENT CELL COUNTS AFTER 30 MINUTES (GLUCOSE SOURCE)

Shear Rate (Inv. Sec)	1% Glucose			3% Glucose			5% Glucose			9% Glucose		
	Cells per mm2	Std. Dev	n	Cells per mm2	Std. Dev	n	Cells per mm2	Std. Dev	n	Cells per mm2	Std. Dev	n
28				3570	400	9	4040	0.08320	5			
46	2970	340	8	2890	290	12	3430	0.14470	14	2630	380	11
71	1710	230	11	2090	300	10	1980	0.11210	13	1840	180	12
120				1490	250	12	1490	0.16240	15			
350							550	0.1370	6			

0.08~0.16

