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STUDIES ON PROTECTIVE ADDITIVES IN CELI CULTURE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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*****

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CHAPTER I
INTRODUCTION

1.1 Introduction

Productions of synthetic drugs, of great therapeutic value, have gained a lot of momentum by the remarkable advances made in the areas of recombinant DNA technology. Research, sponsored mainly by the biopharmaceutical industry and non-profit organizations such as National Science Foundation (NSF), in the area of genetic engineering has made possible the production of foreign proteins in insect and animal cells. These cells can be grown in cultures, leading to the developments in insect and animal cell culture technology. Cultures of these cells have the ability to efficiently produce recombinant proteins with a structure and biological activity that closely resemble the native protein.

The preference for using insect and animal cells over bacterial cells as host is due to the inability of the bacterial host cell to perform post-translational modifications required for the correct biological functioning of many animal proteins. The animal cells are effective hosts for the expression of a gene obtained from a higher eukaryote, because of their ability to recognize properly and efficiently the signals for synthesis, processing, and secretion of the foreign proteins. The proteins can be readily synthesized and secreted into the growth medium, and also the protein folding and disulfide bond formation are usually similar to that of the natural protein. Glycosylation, both N- and O-linked, often occur at normal positions. Other post-translational modifications can occur, and multimeric proteins of single or multiple subunits can be correctly assembled. Insect
cells are also gaining in the field of cell culture. Insect cells act as hosts for the baculoviruses, which are excellent vectors for genetic engineering because of their high expression rate and post-translational processing capabilities. Goosen (1993) had indicated that the typical product yield normally vary from a low of 1-5 mg/L for human interferon to a high of 600 mg/L for β-galactosidase. Insect cell culture may also be employed for producing insect pathogenic viruses for agricultural and forest pest control. As insecticides, the viruses are pathogenic only to the target insects and are not hazardous to the environment. Recombinant baculoviruses have also the potential of replacing the chemical pesticides.

1.1.1 Methods Of Insect And Animal Cell Culture

Presently, a large number of pharmaceutical companies have shown interests in research and development of large-scale cultures of insect and/or animal cells, and this has led to mass production with moderate cost. Commercial production of cells has led to the design of different types of bioreactors. Cell cultures in these bioreactors are, mainly, carried out by two different techniques: suspension cell culture and anchorage dependent cell culture. In the suspension cell culture, the cells are grown suspended in the liquid medium, while in the anchorage dependent cell culture, the cells are grown attached to the surface of the culture vessel or some other solid support.

Large-scale suspension culture based on microbial fermentation technology has clear advantages for the manufacture of insect and animal cell products. The processes are relatively simple to operate and straightforward to scale up. Homogeneous conditions can be provided in the reactor that allow for precise monitoring and control of temperature, dissolved oxygen, and pH, and ensure that representative samples of the culture can be taken. A number of insect cells are grown in suspension cell cultures.
Some of the products obtained from animal cell products grown in suspension cultures are vaccines, interferons, recombinant products, and monoclonal antibodies.

Suspension type cultures can also be applied to anchorage-dependent cells. The cells are allowed to grow on small beads, called microcarriers, and these microcarriers can remain suspended in solution. All the advantages of a typical suspension culture can be applied to a microcarrier culture. Some of the additional advantages of the microcarrier culture is that there is a high surface-to-volume ratio, dependent on the microcarrier concentration. The high surface-to-volume ratio leads to high cell yields. Cell propagation can be carried out in one high-productivity vessel instead of using many small low-productivity units, thus achieving a better utilization and a considerable saving of culture medium. The well-mixed microcarrier suspension culture, in which cells are homogeneously distributed, makes it possible to monitor and control various environmental conditions (pH, dissolved oxygen concentration, and concentration of medium components), thus leading to more reproducible cell propagation and product recovery. Harvesting of cells is relatively easy as the microcarriers settle easily out of suspension. Microcarrier cultures can also be easily scaled up using conventional equipment (fermentors) used for the suspension cultures.

1.1.2 Sparging In Cell Cultures

Sparging, along with impellers, can provide a homogeneous environment in the bioreactors. Sparging is also necessary to supply adequate amounts of oxygen to the cells growing in the culture. Oxygen requirements of insect cells are high (15 to 45 μg O₂/mg cell-hr) (Stockdale and Gardiner, 1976) compared to mammalian cells (1.7 to 19 μg O₂/mg cell-hr) (Streett and Hink, 1978; Glacken et al., 1983). Due to the high oxygen demand of production systems employing these cells in suspension, mass transfer
assisting operations such as agitation and air sparging need to be employed. Pioneering efforts by Hink and Strauss (1976) with *Trichoplusia ni* (TN368) cells in spinner flasks showed that cell growth increases with air sparging the medium with an air flow rate of 5 cm³/min rather than passing a stream of air above the medium surface.

Despite the various advantages of air sparging in large-scale cultures, it also poses a problem in the scaling up of cell cultures. The main technological constraint to large volume cultivation of insect and animal cells in suspension is the supply of sufficient oxygen without aggressive sparging and stirring. Air sparging leads to bubble entrainment, and cell-bubble interactions lead to extensive cell damage in bioreactors. The high hydrodynamic shear forces resulting from the rupture of bubbles lead to cell damage and death. Therefore, in order to optimize the production in a bioreactor, an understanding of the chemistry of the cell-bubble interactions is required.

1.1.3 Causes And Effects Of Shear Due To Sparging

Shear forces result from spatial differences in the levels of momentum across material stream lines in a moving body of fluid. In a stirred bioreactor, cells can encounter a variety of mechanical forces due to collisions with the vessel walls, the agitator, or other objects in the bioreactor. In addition, sparged gas bubbles subject the cells to surface tension forces and also to fluid mechanical forces, resulting from the motion, disengagement and bursting of bubbles, and also from foaming. All of these are collectively called shear. Study of the effect of shear on cells is important as cell physiology can be modulated by the environment. It is also expected that fluid shear stresses will influence the efficiency of the host cell for protein production from recombinant DNA. There is evidence obtained from animal cell culture that shear stresses could modulate protein synthesis (Shuler *et al.*, 1990).
In comparison to microbial cells, insect and animal cells are more sensitive to shear due to their relatively large size (10-20 μm range) and lack of cell wall. Tramper et al. (1986a) conducted experiments to determine the critical shear stress of an insect cell line, *Spodoptera frugiperda* (SF9), and it was found to be of the order of 1.5 N/m². Critical shear stress is the stress at which the number of viable cell declines. For these studies, the cells were grown in a 1 liter round-bottomed fermentor equipped with a marine impeller. The medium contained 0.1% w/v methylcellulose. The effect of shear stress on cell viability was also carried out using a Haake rotaviscometer which confirmed the critical shear stress to be between 1 and 4 N/m². Maiorella et al. (1991) found that the sensitivity of animal cells to hydrodynamic stress is increased in serum-free and low protein media. The critical shear stress of SF9 cells was found as 2.5 N/m² in the presence of Pluronic F68, which is a protective additive.

In suspension cultures with air sparging, cell damage due to cell-bubble interactions have been observed by a number of researchers (Kilburn et al., 1968; Tramper et al., 1986a; Silva et al., 1987; Handa-Corrigan et al., 1987, 1989; Oh et al., 1989, 1992; Kunas et al., 1990a; Bavarian et al., 1991; Chalmers et al., 1991). Significant degree of cell damage was evident in the presence of cell-cell adhesion (when the cells adhere together to form clusters) and also in the case of cell-gas adhesion (when the cells adhere to the air-medium interface). The effect of airflow on viability of SF9 cells was studied by Tramper et al. (1986a) using a bubble column reactor (height 0.18 m, inside diameter 0.035 m). The experiment was performed at different gas flow rates and using different air spargers. They found that cell death was proportional to the air flow rate, but the effect of bubble size on cell damage was less pronounced. The loss of viability due to direct air sparging was explained in terms of an adherence of cells to the bubble/liquid interfaces and rupturing of bubbles at the surface of the suspension. The
estimated shear stress an adhered cell could experience in the event of a bubble rupture is 625 N/m² which is roughly two orders of magnitude above the critical value of 1 N/m². Alternative oxygenation strategies, such as membrane bioreactors or diffusion of gas through semi-permeable tubing, were recommended. It has also been shown by Garcia-Briones et al. (1994) by computer simulations that very high levels of shear stresses are obtained during the bubble rupture process, and that these are functions of the size of the bubbles. The cells attached to these bubbles experience high levels of shear stresses and the magnitudes of these shear stresses are sufficient to damage the attached cells. Tramper and Vlak (1986b) came to similar conclusions with animal cells.

Handa-Corrigan et al. (1987) showed that cell viability decreased with increasing bubble frequency and decreasing bubble size. They also suggested that the maximum damage occurs at the surface, which is the region of highest shear and turbulence and where bubble disengagement occurs. Handa-Corrigan et al. (1989) found that bubble column bioreactors with large height to diameter ratios ensure better cell growth due to reduced time spent by the cells in the bursting bubble zone. It was suggested that cell damage in sparged bioreactors occurs due to:

(i) damage due to a physical shearing effect in the draining liquid films around the bubbles;
(ii) rapid oscillations caused by bursting bubbles;
(iii) physical loss of the cells in the foam.

Studies made by Jobses et al. (1990) also corroborated the results obtained by Handa-Corrigan et al. (1987, 1989). It was found that the cell death rate is a linear function of the specific gas flow rate, and cell death occurs mainly at the surface during bubble escape. They also showed that the cell damage is not dependent on the culture height and cell damage does not take place at the sparger site. Evidence of the
detrimental effect of gas sparging on the cells was also reported by Oh et al. (1989), when they showed that there was a reduction in the total cell concentration and cell viability with sparging in the bioreactors. They also showed that cell damage in bioreactors increased as the volumetric flow rates increased, and detrimental effects of bubbles increased with decrease in size of the bubbles. Kunas and Papoutsakis (1990a) have shown that two fluid mechanical mechanisms can cause cell damage and growth retardation in agitated bioreactors. The first is associated with vortex formation, bubble entrainment, and breakup. In the absence of vortex formation and bubble entrainment, cells can be damaged only with extremely high agitation rates (700 rpm in a 1-2 liter bioreactor).

Reports on the hydrodynamics of the bubble breakup in relation to cell damage were made by Chalmers et al. (1991) and Bavarian et al. (1991), when they used high-speed video and microscopic system to visualize cell-bubble interactions. Considering their observations, they proposed the following mechanisms that can cause cell damage in sparged and agitated bioreactors with bubble entrainment:

(i) physical loss of the cells attached to the bubble into the foam layer;
(ii) cell damage occurs due to the shear stress experienced by the cells when they adhere to the bubble surface and rise through the medium along with the bubble;
(iii) cells attached to the bubble are damaged when the bubble film ruptures;
(iv) cell damage occurs in the boundary layer flow region in the bubble cavity after the bubble film has ruptured.

Similar results have also been found in the case of microcarrier cell cultures. Though microcarriers are used for the culture of a number of animal cell lines, the cells on microcarriers are especially susceptible to damage from fluid mechanical forces. In addition to the lack of cell wall, and the relatively large size, these cells lack individual
cell mobility. Anchored cells cannot rotate or translate, and therefore, they cannot reduce the net forces and torque experienced upon exposure to fluid-mechanical forces.

Studies were conducted to evaluate the shear sensitivity of the cells attached to microcarriers. In microcarrier cultures, sparging is required for gas-liquid mass transport (oxygenation) and liquid-phase mixing (Croughan and Wang, 1991). When anchored cells are exposed to sufficiently high shear stresses, cell removal from the growth surface will generally become significant, and detachment of cells from the solid support results in cell death. Crouch et al. (1985) observed extensive cell removal for shear stresses in the range of 30 dynes/cm² or greater in experiments with animal cells (BHK, Vero, and MRC5) grown on plastic or glass slides. They also reported that the viability of the cells remaining attached were greater than 90%. Viggers et al. (1986) observed extensive cell removal from the growth surface for shear stresses of 128 dynes/cm². Croughan and Wang (1989) found that removal of FS1 cells from microcarriers grown in 125 mL spinner flask increases with increased agitation and thus removal of cells from microcarriers appears to be due to hydrodynamic forces. The adverse effects of sparging on the cell viability in microcarrier cultures were also shown by Aunins et al. (1986). Evidence exists that the viable cell concentrations were lower than in the controls and the damage was evident even though the superficial gas velocity was only 0.01 cm/sec and there was no significant foam formation.

From the results obtained by the different research groups, mentioned above, for different types of cultures (suspension culture and microcarrier culture), it can be said that the cells are definitely damaged in the presence of air sparging. The cells tend to adhere to the gas-liquid interface and experience high levels of shear stresses in the event of bubble rupture. Therefore, a way of preventing cell damage will be to prevent the cells from adhering to the air-medium interface.
1.2 Objective Of The Study

Cell damage in bioreactors is definitely a problem and one of the ways of solving that problem is the addition of protective additives to the culture medium. The objective for the study conducted can be classified under three broad categories. The first part deals with understanding of the mechanism by which the protective additives prevent cell damage in a typical bioreactor. The second part deals with the identification of the most effective protective additive among all the additives used for the study. The last part of the study deals with understanding the problem of cell damage due to air sparging. Evidence exists that cell damage is mainly due to cell-bubble interactions. Thermodynamic study has been conducted to predict the feasibility of the occurrence of cell-gas adhesion under a particular condition. This is also useful in understanding how the protective additives prevent cell-gas adhesion.

After a careful review of the existing literature on the protective additives, a proposal to explain the action of the protective additives was formulated. Experiments were conducted to observe the change in the degree of cell damage due to bubble rupture in the presence of different protective additives. This set of experiments was also useful in identifying the most effective protective additive in preventing cell damage in the presence of sparging in bioreactors. Since it is already known that cell-gas adhesion is responsible for cell damage, experiments were conducted to investigate the change in the degree of cell-gas adhesion in the presence of protective additives.

A thermodynamic relationship was developed to predict the possibility of adhesion of cells to the air-medium interface based on the change in free energy for the process. The change in free energy ($\Delta F^{adh}$) is dependent on the interfacial tensions ($\gamma$) of the phases involved. For the conditions existing in a bioreactor with air sparging, there are three phases: cells (c), liquid culture medium (l), and surrounding vapor (v).
Experiments were conducted to determine the various interfacial tensions involved. Substituting these values in the proposed thermodynamic equation, the $\Delta F_{\text{adh}}$ for a particular set of conditions was determined. These results will predict the feasibility of cell adhesion to the air-medium interface. A negative $\Delta F_{\text{adh}}$ corresponds to a feasible process, while a positive $\Delta F_{\text{adh}}$ indicates a thermodynamically unfavorable process. In order to prevent cell-gas adhesion, the value of $\Delta F_{\text{adh}}$ should be changed from a negative value to a positive value, and manipulation of the different interfacial tensions can bring about such changes. Investigations have been conducted to study whether the presence of the protective additives in the liquid medium would affect the interfacial tensions. Both theoretical and experimental studies have been conducted to prove the validity of the proposed thermodynamic equation.

The different types of experiments conducted include measurements of liquid-vapor interfacial tensions of the different liquid media, with and without protective agents. Out of the three different interfacial tensions involved, only one can be determined experimentally. The remaining two interfacial tensions (cell-vapor interfacial tension, and cell-liquid interfacial tension) have been calculated from semi-empirical equations for which data on contact angles made by the liquid on the cell surfaces are required. Experiments were conducted to measure these contact angles. Different methods were used both in the measurement of liquid-vapor interfacial tension, and also in the measurement of contact angles. From these results, speculations were made regarding the interaction between the protective additives and the cell membrane. Separate experiments were also conducted to measure the change in the lipid composition of the cells by investigating the lipid composition of cell extracts with thin-layer chromatography. This is because any interaction between the lipid bilayers of the cell membrane and the additives would bring about a change in the interfacial tensions, thus
affecting the $\Delta F^{\text{adh}}$ of the process.

1.3 Protective Additives

The design of the bioreactor can be suitably adapted to the requirements of the shear sensitivity of insect and animal cells, and a large number of studies have been conducted in this area (Malinowski and Daugulis, 1993). However, several researchers have also observed that instead of complicated designs of bioreactors, cell damage can be reduced appreciably by the addition of certain polymeric additives, also known as protective agents, to the culture medium. The commonly used protective additives for cell cultures include serum, pluronic polyols, various derivatized celluloses and starches, protein mixtures, dextrans, polyethylene glycol (PEG), polyvinyl alcohol (PVA), and others (Papoutsakis, 1991).

Researchers (Hink et al., 1979; Smith et al., 1987; Garcia-Briones et al., 1992) have also observed that the presence of these protective additives reduces cell-cell adhesion and cell-gas adhesion. Hink and Strauss (1976) found that the addition of methylcellulose (0.1% w/v) improved cell density of a culture of TN368 cells in spinner flasks by reducing cell clumping. Further studies by Hink et al. (1979) showed that addition of 0.3% methylcellulose (Methocel Dow 65 HG) to the medium (Grace's tissue culture medium) during the culture of TN368 cells in a spinner flask reduced aggregate formation and also decreased the percentage of cell damage. In 1982, Hink found that the growth of TN368 cells in a 3 liter fermentor stopped at a stirrer speed of 220 rpm in medium containing 0.1% methylcellulose, but continued in 0.3% methylcellulose at the same agitation rate. This also showed that the protective action is dependent on the concentration of methylcellulose in the medium.
Garcia-Briones et al. (1992) have reported, based on their visual observations, that in the presence of Pluronic F68 in the culture medium (TNMFH) for the SF9 cell line, cell adhesion (both cell-cell adhesion and cell-gas adhesion) was minimum. Similar observations were also reported by Smith et al. (1987) when they observed that Pluronic F68 reduced adherence of the sickle erythrocytes to endothelial membrane. It was also observed that decrease in cell damage was proportional to decrease in aggregate formation. The above evidence indicates that these protective agents prevent cell damage essentially by preventing cell adhesion and thus protecting the cells from the high levels of shear stresses.

1.3.1 Mechanisms Of Protection

Different mechanisms have been proposed to explain the protective action of the additives in the culture medium during cell culture (Michaels et al., 1991; Papoutsakis, 1991). It should also be kept in mind that the protection mechanism offered by the protective additives are also dependent on the cell type. The three most commonly held views are:

(i) the additives have a nutritional effect on the cells, which is the metabolic biological mechanism;

(ii) the additives interact with the cell membranes, thus strengthening the cell membrane by forming a protective layer, and this is the fast-acting biological mechanism;

(iii) the cell membrane is not affected by the presence of the additives and the protective action of the additives is entirely physical in nature.

Serum, which is added to the culture medium to enhance cell growth and acts mainly as a nutrient, was found to act as a protective agent against fluid-mechanical
damage in bioreactors also. It was reported by Michaels et al. (1991b) that increasing concentrations of fetal bovine serum (FBS) have an increasing protective effect on the hybridoma cells (CRL 8018) and the protective effect of FBS was evident both after prolonged and short (approximately 1 hour) exposure. However, viscometric experiments with 5 to 10 percent FBS showed that FBS did not affect the shear sensitivity of the cells. This led them to believe that the protection mechanism of FBS is to a large extent physical in nature. However, they did not ignore the biological mechanism of shear protection and their conclusion was that the protection mechanism of FBS may be both physical and biological (metabolic). It was shown by Papoutsakis et al. (1990) that the ability of the cells to resist shear is related to the integrity of the actin micro-filaments of the cytoskeleton and to the cells' ability to maintain an active state of energy metabolism. The metabolic component of the protective effect of FBS may be due to such contributions.

Ramirez and Mutharasan (1990, 1992) showed that the nutritional and the protective aspects of serum are independent of each other. The nutritional effect was seen by comparing the viability indices within the static and agitated cultures at increasing serum concentrations. To uncouple the nutritional effect of the serum from its protective effect, the initial specific growth rates and death rates were determined in the static and agitated batch cultures. They evaluated the plasma membrane fluidity (PMF) of the cells and correlated PMF with the shear sensitivity of a particular cell line. They stated that in laminar flow, increased membrane fluidity corresponded to increased shear sensitivity, and a decrease in the membrane fluidity makes the cells more shear-resistant. They found that the PMF of the hybridoma cells decreased in the presence of serum, and this might account for the decrease in cell damage in the presence of serum in the liquid medium. They also showed that increasing the cholesterol content in the culture medium reduced
Kunas and Papoutsakis (1989, 1990b) showed that increase in cell growth rate in agitated cultures results from increasing the serum concentration from 5% to 10%. They also showed that CRL 8018 cells became more resilient within a very short period after serum was added to the growth medium. This led them to conclude that the protective action was not primarily nutritional, but rather a fast-acting mechanism, as the time period is not sufficient for the cells to metabolize the additives. However, they did not rule out the nutritional effect of serum. They also found that the small increase in viscosity due to the addition of serum to the liquid medium is not responsible for the protective action. This is corroborated by the fact that even an increase in viscosity of the medium by 50% to 100% by the addition of high molecular weight dextrans did not prevent cell damage in bioreactors. So, from the findings of the various research groups it can be stated that the protection mechanism of serum is both biological (metabolic and fast-acting) and physical.

Though the presence of serum has a nutritional effect on the cells, the protection resulting from the metabolic biological mechanism is not significant for most of the protective additives, as it would be highly unlikely for the cells to metabolize polymeric substances, like cellulose derivatives, pluronics, and others. Mizrahi (1984) showed conclusively that over 99% of the polymers (carboxymethyl cellulose, hydroxyethyl starch and pluronics) added to the culture medium were not consumed by the cells during the course of the experiments.

A second school of thought believed that the protective additives interact with the cell membrane and as a result of this interaction, the cells become more shear resistant. Evidence of adsorption of the protective additives on to the cell membrane was provided by Murhammer et al. (1988, 1990a) when they studied the effect of Pluronic F68 on
sparged cultures of the insect cell-line, SF9. They proposed that the pluronics interact with the cell membrane, and their inference was based on trypan blue dye exclusion test and other experimental evidences. The presence of Pluronic F68 in the medium inhibited the uptake of trypan blue dye by cells that would normally absorb the dye in the absence of the additive. Similar proposals were also put forward by Kilburn and Webb (1968) and Mizrahi (1984). Kilburn and Webb (1968) have suggested that Pluronic F68 exerts its protective effect through the formation of an interfacial structure of adsorbed molecules on the cell surface, presumably as a result of the surfactant nature of the polymer. Mizrahi (1984) found that the uptake of glucose by the cells decreased in the presence of carboxymethyl cellulose or hydroxyethyl starch. This led him to conclude that these polymers protect the cells by forming a protective layer. Mizrahi (1984) also noticed a lowering of the surface tension of the medium in the presence of pluronics.

In 1990, Goldblum et al. reported the effect of protective additives on two insect cell lines, TN368 and SF9, when the cells were subjected to various levels of well-defined laminar shear stress in a modified Weissenberg rheogoniometer. It was observed that cells were more resistant to laminar shear stress in the presence of protective additives, and there was a significant decrease in the rate of cell lysis. The protective action of Pluronic F68 was found to be dependent on concentration and it was seen that the resistance of SF9 cells increased significantly when the concentration of Pluronic F68 was increased from 0.2% to 0.3%. In the case of TN368, Goldblum et al., (1990) found that for a given weight percentage, greater protection is obtained with higher molecular weight forms of Methocels (methylcelluloses and hydroxypropyl methylcelluloses) and that hydroxypropyl substitution increased the protection for a given molecular weight. However, with SF9 there is not as great an increase in protection with hydroxypropyl substitution. They also found that the amount of the protection from laminar shear stress
was the same regardless of whether the cells were grown containing protective additives in TNMFH medium or resuspended in medium containing the additives. From the experimental results obtained, it was concluded that the polymers were adsorbed on to the cell membrane and the adsorbed layer provided protection against the laminar shear stresses.

Ramirez and Mutharasan (1992) have also supported the idea of direct interaction between the additives and the plasma membrane of the cells with their experiments in which the serum was present in the liquid medium. Smith et al. (1987) reported that the rigidity of the sickle erythrocytes cells were reduced in the presence of Pluronic F68, and from these results they concluded that there was an interaction between the additive and the cell membrane. In studies unrelated to cell culture, Cudd et al. (1989) showed that dextrans of molecular weight higher than 40,000 inhibited low pH hemolysis of erythrocytes. They also showed by electron microscopy that dextrans of high molecular weight (150,000 or higher) form a tight association with the cell membrane. Clarke et al. (1992) also showed that Pluronic F68 increases cell survival during syringe loading, a novel technique for introduction of normally impermeant macromolecules into the cytosol of living mammalian cells, and this indicated that Pluronic F68 has the capability of altering the physical properties of the plasma membrane.

Further evidence of interaction between the protective additives and the cell membrane was provided by Murhammer et al. (1990b). As a result of testing of a number of pluronics and reverse pluronics, they found that the protective action of the additives is directly proportional to their hydrophilic-lipophilic balance (HLB) number. The HLB number has been defined as the ratio of the molecular weight of the hydrophilic portion of the molecule to that of the hydrophobic portion. Surfactants with low HLB values are more hydrophobic (more oil soluble) than surfactants with higher HLB values,
which are relatively more hydrophilic. It has been observed that surfactants with very low HLB values are used to lyse cells. Triton X100 (HLB value of 13.5, Helenius et al., 1975) and Pluronic L61 (HLB value 1-7, Murhammer et al., 1990b) are membrane-disrupting agents. The process of cell lysis by surfactant molecules can be divided into five steps (Helenius et al., 1975): (i) surfactant molecules adsorb onto the membrane; (ii) surfactant molecules penetrate into the membrane; (iii) within the membrane, the surfactant molecules induce a change in molecular organization; (iv) this change leads to an alternation in the permeability and in the osmotic equilibrium; and (v) this finally leads to cell damage. So cell lysis is brought about by a strong interaction between the surfactants and the cell membrane. On the other hand, it has been shown that surfactants with higher HLB values do not affect the cells and can even be used to prevent cell damage (Pluronic F68 of HLB value >24 prevents cell damage; Murhammer et al., 1990b). But along with the HLB value, due considerations should be given to the molecular structure of the hydrophobic portion (poly(oxypropylene)) of the pluronic and reverse pluronic polyols. It has been experimentally observed that pluronics with a saturated hydrocarbon chain interact more readily with the cell membrane, causing cell lysis, than pluronics having an unsaturated hydrocarbon chain, even though the HLB values are in the same range. The above two sets of observations led them to conclude that it is the hydrophobic portion of the pluronics that interacts with the membrane. It was also shown that the size of the poly(oxyethylene) molecules is directly proportional to the protection provided by the surfactant. It was observed that poly(oxyethylene) molecules of average molecular weight of 2000 provided less consistent protection in the agitated, sparged bioreactor than did the poly(oxyethylene) molecules of molecular weight 8000. According to them, the hydrophilic (poly(oxyethylene)) portion is available to interact with the outer surface of the cell membrane, either by adsorption or through
hydrogen bonding between the poly(oxyethylene) oxygen and lipids and/or proteins in the cell membrane. The poly(oxyethylene) oxygen can also form hydrogen bonds with the water molecules, creating an ordered array of water molecules many layers thick around the cell. Another possible mechanism of protection is that the pluronics can also interact with the air-liquid interfaces. The presence of pluronic polyols and an associated hydration sheath on the surface of both cells and bubbles could provide a steric barrier to damaging cell-bubble interactions. The reduction of aggregate formation can also be explained by the presence of this hydration sheath.

Handa-Corrigan et al. (1989) put forth a different theory explaining the cause of cell damage in the growth medium. According to them, cell damage is either due to shearing during liquid drainage from the bubble film, or it may be due to oscillatory disturbances caused by the rupturing bubbles. They suggested that the presence of Pluronic F68 forms a stable foam layer and thus protects the cells from the damaging shearing effects of film drainage and/or bursting bubbles. Interactions between the cells and the air-liquid interface were also not observed in the presence of Pluronic F68. So it can be said that the mechanism proposed by Handa-Corrigan et al. (1989) is based on the concept that the protective action is entirely physical in nature.

Michaels et al. (1991b) studied the effect of serum, Pluronic F68 and polyethylene glycol (PEG) on the growth of CRL 8018 cells. Their experimental findings regarding the protective effect of PEG and Pluronic F68 corroborated the theory proposed by Handa-Corrigan et al. (1989). Michaels et al. (1991) did not support the concept of incorporation of the protective additives in the membrane. Their deduction was based on the result that addition of Pluronic F68 and PEG did not affect cell growth in static culture, which indicated that the cell membranes were not made strong as a result of adsorption of the additives. Adding to the complexity of the situation, Murhammer et al.
(1990a) found that the protective action of Pluronic F68 was evident even in the absence of foam. It was inferred that stabilization of the foam layer, as proposed by Handa-Corrigan et al. (1989), was not the only reason for the protective action. Jobses et al. (1990) showed that the protective effects of Pluronic F68 were observed in experiments on bubble-free fluid mixing, along with sparging experiments. This led them to conclude that the protective action is mainly due to a direct interaction of the additives on the cells, possibly by the formation of a stagnant fluid film around the cells.

1.3.2 Proposed Mechanism Of Protection

After a careful review of the mechanisms proposed to explain the protective action of the additives, it was suggested (Chattopadhyay et al., 1995a) that the presence of the protective additives affects the interfacial properties, such as interfacial tensions between the different phases involved in a bioreactor. Change in the interfacial properties will change the manner in which two phases interact. It has already been identified that cell damage in the presence of sparging is due to cell-bubble interactions. A change in the interfacial tension will influence the degree of cell-gas adhesion and this would affect cell damage. A thermodynamic explanation (Chattopadhyay et al., 1990b) has been provided to illustrate the reduction in cell-gas adhesion in the presence of protective additives. The mathematical relationship explains the occurrence of cell-gas adhesion in terms of change in free energy of the process which is dependent on interfacial tensions. It was also suggested that change in the interfacial tensions will predict the feasibility of the process of cell-gas adhesion.
1.4 Cell Adhesion

The phenomenon of cell-cell adhesion was reported by Telling and Elsworth (1965) during the culture of Baby Hamster Kidney cells (BHK21) and also by Hink and Strauss (1979) during the suspension culture of the cabbage looper (TN368) cells in a spinner flask. The phenomenon of cell-gas adhesion during suspension culture of TN368 and SF9 cell lines with air sparging was reported by Bavarian et al. (1991). Microscopic and high-speed video technology was used to study adhesion of the cells to the bubbles rising through the medium and also to the air-medium interface at the top of the column. In 1992, Garcia-Briones et al. reported their visual observations that insect cells (SF9) did not adhere to the air-medium interface when Pluronic F68 was present in the medium TNMFH. However, cell-gas adhesion was evident in the same liquid medium in the absence of the protective additives. Since cell damage in bioreactors was reduced in the presence of the protective additives, it showed conclusively that at least one of the mechanisms for prevention of cell damage is the prevention of cell adhesion to the bubble film.

In 1970, Parker and Barsom reported that the chemical composition of the surface microlayer, which is the thin layer of liquid at the air-liquid interface, is very much different from that of the subsurface water. It has also been noted that the compositions of the surface monolayer, the surfaces of bubbles rising through water, and the jet and film drops formed when bubbles burst at the air-water interface are closely interrelated and interdependent (Kjelleberg, 1985). The bubbles tend to collect any surface-active material present in the liquid, and concentrate these materials at the surface film. A large amount of hydrophobic substances, especially microorganisms, tend to accumulate in this layer. Blanchard and Syzdek (1982a) have found that the bacterial concentrations in the jet drops from bursting bubbles may be 1000-fold greater than concentrations in the bulk.
Bubbles breaking at the air-water interface act as effective microtomes, skimming material from the bubble surface and ejecting it as jet drops into the atmosphere (MacIntyre, 1968, 1970). As the bubble reaches the surface, the surface free energy of the bubble is converted into kinetic energy causing a burst of jet and film drops, which are smaller and more numerous, into the atmosphere.

The phenomenon of bacterial cell adhesion to the air-liquid interface has been studied widely (Dahlback et al., 1981; Blanchard et al., 1982b; Hermansson et al., 1982, 1983; Kjelleberg et al., 1980, 1985). It has been found that the possible contributing factors for cell-gas adhesion are various, including electrostatic forces, van der Waals' forces, chemical interactions between the cell surface and the air-liquid interface, and hydrophobic interactions. The electrostatic attraction can be due to opposite charges between cells and substrata. It should be pointed out that the surface charge is not uniform, and the substratum can also take up many of the properties of the adsorbed proteins resulting from the presence of proteins in media (usually serum). The distinction between nonspecific physical and specific molecular interactions is purely semantic, since on the molecular level, chemical interactions do involve physical forces (in addition to van der Waals and dipolar interactions, there are also hydrogen-bonding and hydrophobic interactions). Hydrophobic interaction leads to the accumulation of amphipathic substances at the air-liquid interfaces. The aggregation of the hydrophobic groups (hydrophobic groups of the amphipathic molecules and the air) is mainly due to the tendency of the non-polar groups to avoid water. Studies by Dahlback et al. (1981) have shown that the accumulation of bacteria on the air-liquid interface is directly proportional to the hydrophobicity of the bacterial cell surface, and a higher proportion of hydrophobic bacteria is found at the air-water interface than in the subsurface water. Since hydrophilic-hydrophobic interactions and also electrostatic interactions are found to be
responsible for the attachment of bacteria to the air-liquid interface, bacteria-gas adhesion is considered to be non-specific in nature (Kjelleberg et al., 1985).

The non-specificity of the bacterial cell adhesion to a substrate is also supported by the experiments conducted by Absolom et al. (1983). They studied the phenomenon of bacterial cell adhesion to various polymeric surfaces, and proposed a relationship based on thermodynamic considerations, for predicting the possibility of the cell-solid adhesion. According to Absolom et al. (1983), adhesion of the cells to the solid surface is dependent on the interfacial tensions between the three interacting phases; the solid (S), the bacteria (B) and the surrounding liquid (L).

\[ \Delta F_{\text{adh}} = \gamma_{\text{BS}} - \gamma_{\text{BL}} - \gamma_{\text{SL}} \]  

where \( \Delta F_{\text{adh}} \) is the change in free energy for the process, and \( \gamma \) is the interfacial tension. Various experiments were performed to show the validity of the proposed thermodynamic relation. Schakenraad et al. (1988) have also suggested a similar thermodynamic approach to measure the non-polar interactions between cells and substratum, based on interfacial free energy. The interfacial free energy of adhesion has been defined as:

\[ \Delta F_{\text{adh}} = \gamma_{\text{SC}} - \gamma_{\text{SL}} - \gamma_{\text{CL}} \]  

where the terms on the right side of the above equation are respectively, the substratum-cell (SC), the substratum-liquid (SL), and the cell-liquid (CL) interfacial free energies. The contributions from the different interfacial tensions can be assessed experimentally, based on a contact angle measurement (Absolom et al., 1983). A high value for the surface free energy has been shown to be positively correlated with cell adhesion and
1.5 Lipid Structure Of The Cell Membrane

1.5.1 Definition Of Lipids

Lipids are an extremely heterogeneous group of molecules that participate in a wide variety of cellular functions. The term “lipid” has been used to define a wide variety of natural products, like fatty acids and its derivatives, steroids, terpenes, carotenoids and bile acids. A more specific definition restricts the lipids as fatty acids and their naturally occurring derivatives (esters or amides) and to compounds closely related biosynthetically to fatty acids. All of these products have a common behavior: they are readily soluble in organic solvents, like diethyl ether, hexane, benzene, chloroform or methanol. The two main features of lipids that affect their solubility in organic solvents are: (i) the nonpolar hydrocarbon chains of fatty acid or other aliphatic groups; and, (ii) any polar functional groups such as phosphate or sugar residues. Lipids that contain no polar groups are highly soluble in hydrocarbon solvents, such as hexane, benzene, and also in slightly more polar solvents, like chloroform or diethyl ether. However, they are insoluble in methanol which is more polar. The solubility of the lipids in the polar solvents increases with the decrease in the chain length of the fatty acid residues, and/or with the increase in the polar functional groups.

The main lipid classes consist of fatty acids linked by an ester bond to the trihydric alcohol, glycerol, or to other alcohols, such as cholesterol, or by amide bonds to long-chain bases (sphingoids or sphingoid bases), or on occasion to other amines. In addition, they may contain alkyl moieties other than fatty acids, such as phosphoric acid, organic bases, carbohydrates, and others. Classifications of lipids are varied, and a way of subdividing them is to group them as: (i) simple lipids, and (ii) complex lipids. This
classification is based on the number of products obtained after hydrolysis of the lipid. Simple lipids are those which on hydrolysis yield at most two types of primary products, while complex lipids are those which yield three or more primary hydrolysis products. An analysis of the lipids from a given source would involve separation of the lipids in different categories, followed by their identification.

1.5.2 Lipids In Biological Membranes

Lipids have important roles in maintaining cell and bio-membrane structure. The lipid bilayer membranes are essentially elastic (deformable) structures. Membranes are mainly composed of lipids, proteins, and sugars. Lipids are generally present as lipoproteins and liposaccharides. The lipids in the membranes can be subdivided into two groups, based on their hydrocarbon tail. The amphipathic lipids are the phospholipids which includes glycolipids, phosphoglycerides, and sphingolipids. All of these lipids have ionic or polar heads, which can combine with the water, and nonpolar hydrocarbon tails. The common hydrocarbon tails of the amphipathic lipids are esters of long chain fatty acids. These acids usually contain an even number of carbon atoms. Both saturated and unsaturated fatty acids can be present in the membrane, and the fluidity of cell membranes is very much dependent on the ratio between the saturated and the unsaturated fatty acids. The second class of lipids are the sterol family, which is also amphipathic in nature, however, in this case, the nonpolar tail is made up of the steroid ring system. Lipids from this category are found in insect and animal cells, but not in bacterial cells.

The main characteristic of the lipids is that they form a bilayer matrix held together by a hydrophobic interaction with the surrounding polar environment. Lipids can form micelles (spherical or cylindrical) or bilayers based mainly on the area of the
hydrophilic head group and the chain length of the hydrophobic tail. Membrane lipids are amphipathic molecules. Phospholipids and glycolipids that contain some unsaturated fatty acids spontaneously form lipid bilayers, in which the polar heads are exposed to the aqueous phase and the hydrocarbon tails are buried in van der Waals' contact with one another. Another property of the lipid bilayer membrane is its selective permeability. However, it has been observed that the permeability of the membrane can be increased selectively by the addition of small amounts of various substances. Several antibiotics and other cation-complexing molecules have been found to markedly increase passive ion transport across the membrane. Further evidences of increase in permeability by the addition of various substances will be shown later.

The gross organization of the biological membrane is given by Singer and Nicholson in their fluid mosaic model. According to them, most of the membrane phospholipid and glycolipid molecules are in the bilayer form. The constituents of biological membranes are free to diffuse laterally, rotate about their major axes and oscillate about the normal to the plane of the membrane. However, they are not free to rotate from one side of the membrane to the other. Traditionally, these various motions as well as the degree of packing of the components of the membrane have been described as the "membrane fluidity". Ramirez and Mutharasan (1990) have determined the change in this membrane fluidity due to the presence of benzoyl alcohol, temperature, and cholesterol in the culture medium.

The major classes of lipid commonly found in animal cell membranes are glycerophospholipids, sphingolipids, and sterols (Ofek et al., 1985). In most animal cells, the most abundant lipids are glycerophospholipids. Glycerophospholipids consist of a glycerol moiety, each hydroxyl group of which is esterified to a fatty acid. The most abundant phospholipid found in cell membranes is phosphoglycerides, which is derived
from a phosphate ester of glycerol. They are the major components responsible for the bilayer organization of the membrane. A second group of phospholipids contains the long chain amino alcohol sphingosine as the central unit. These sphingolipids are the second major lipid component of animal cell membranes. The most common sterol present in the animal cell membrane is cholesterol. It can be found in the free state, where it has a vital role in maintaining membrane fluidity, and also in esterified form, such as cholesterol esters.

In tissues, the structural lipid components, such as the phospholipids, tend to be rather constant in composition so meaningful comparisons between different organs can often be made (Christie, 1987). On the other hand, the proportions of the simple lipids, especially the triacylglycerols, can vary greatly according to the dietary or physiological state of the animal. The fatty acid composition of each lipid in a tissue is frequently distinctive and can vary markedly between species. All glycolipids of the cell membrane are constituents of the outer half of the bilayer, where they comprise 30-60% of the lipid content (Ofek et al., 1985). The ceramide group is responsible for anchoring the molecules to the membrane by intercalating within the lipid bilayer while the oligosaccharide chain extends to the outer surface. The net negative charge of the animal cell surface is mainly due to the presence of sialic acid in glycolipids.

1.5.3 Hydrophobic Effect In Lipids

Lipids, like synthetic surfactants, can form micellar structures, and they also spontaneously form lipid bilayers, which is the basis of biological membranes. These phenomena can be attributed to the hydrophobic effect, which is probably the single most important factor in the self-assembly of biological membranes. The interactions between lipids and proteins are also guided by the hydrophobic effect, as both proteins and lipids
are amphipathic molecules. In the majority of the lipid molecules in the biological membrane, two hydrocarbon chains are attached to a single head group. In these lipid bilayers, the hydrophobic tails lie inside the bilayer, while the hydrophilic heads contact the aqueous solution. In case of the phospholipids, which are the most abundant lipids found in the membranes, the polar head is highly solvated. However, the nonpolar tails should avoid contact with the water molecules so as not to create an unfavorable entropy change. This is done by the formation of the bilayer with the hydrophobic end pointing inwards.

A cell is essentially a plasma membrane - encapsulated vesicle, although it is usually not a perfect sphere owing to other structural contributions to its shape. The formation of vesicles by the phospholipids is favored over a planer bilayer because exposed hydrocarbon tails, which occur at the periphery of a planer sheet of phospholipid, are not present. In vesicular structures, no hydrophobic groups need to be exposed to water molecules. In addition, most naturally occurring phospholipids prefer to form vesicular bilayers instead of micelles in water solution because more efficient packing of the molecules can take place in the bilayer vesicle. Phospholipid bilayers are relatively impermeable to most hydrophilic substances because of their hydrophobic interior. This is a property of a biological membrane, unless a transport system recognizes the hydrophilic molecule. However, water is a major exception.

When phospholipid is added to water, very few of the lipid molecules exist freely in solution as monomers. Instead, a film of phospholipid tends to form on the water-air interface and this film is a monolayer of phospholipid such that the polar head groups are in contact with water, while the hydrocarbon tails extend up into the air phase. When more phospholipid is added to the solution, saturating the air-water interface, other assemblages of phospholipids are formed, including micelles and bilayers. Both these
structures maximize hydrophobic and van der Waals interactions between fatty acyl chains, effectively excluding water from their vicinity and allowing the polar head groups to interact with the water molecules. Monolayers, micelles, and bilayers are the favored forms of phospholipids in aqueous solution because their formation results in an increase in entropy, resulting from the fact that the water molecules need not order themselves around the hydrophobic hydrocarbon tails of the phospholipid monomer.

Effect of the environment on the membrane is shown by the incorporation of cholestérols in membranes. Though cholesterol molecules do not form bilayers, they are easily incorporated in the bilayer structure formed by other lipids. Cholesterol is extremely insoluble in water and at a concentration higher than $10^{-6}$ M, phase separation occurs. Cholesterol readily enters into micelles formed by phospholipids, and even equimolecular amounts of phospholipids and cholesterol have been found in mixed micelles. The retention of hydrocarbon in artificial membranes and the incorporation of cholesterol in phospholipid bilayers are consequences of the non-specific nature of the hydrophobic force. Any amphipathic substance should be able to enter into phospholipid micelles.

Another interesting observation made is the use of detergent in disrupting bilayer structure. When a detergent is progressively added to any collection of lipid molecules, equilibrium between the various amphipathic molecules is established. In the event of addition of excess detergent to a system of lipid bilayers, the detergent forms micelles containing small amounts of lipids. This brings about a disruption of the lipid bilayers. Formation of micelles by the detergent molecules is also due to the hydrophobic effect.
CHAPTER II
MATERIALS AND METHODS

2.1 Materials Used

2.1.1 Cell Lines

Both insect and animal cell lines have been used for this present study. The cell lines used for the study can be sub-cultured indefinitely. They are capable of growing either in suspension or attached to a substrate. Suspension-type cultures can also be done with the anchorage dependent cells where they are allowed to attach to the microcarriers, and these microcarriers can be suspended in the liquid medium.

Two types of insect cell-lines have been used: Spodoptera frugiperda (SF9) cells and Trichoplusia ni (TN368) cells. SF9 cells were derived and isolated from the SF21AE cell line and SF21AE cells were isolated from the pupae ovaries of the fall army worm. TN368 cells were isolated from the pupae ovaries of adult cabbage looper. Both these cell-lines belong to the insect order Lepidoptera, which is commonly used to produce proteins by recombinant DNA techniques, and are more applicable for large-scale cultures. The SF9 cells are primarily round with a diameter of approximately 20 μm. The TN368 cells have a diameter ranging from 10 to 50 μm and possess protoplasmic extensions. The doubling time of these cells is approximately 16 hours. Both these cells can be grown in suspension cultures. These two cell lines were obtained from Dr. Fred Hink (Department of Entomology, The Ohio State University, Columbus, OH).

Three different animal cell-lines have been used: hybridomas (HT24, obtained from Dr. Richard F. Mortensen, Department of Microbiology, The Ohio State University, Columbus, OH), CHOK1 (Chinese Hamster Ovary, ATCC-CCL 61) and N-VERO
Hybridomas result from cell hybridization of B or T lymphocytes with suitable indefinite life-span, cell-like lymphomas, myelomas, etc. (transformed cells). Parental CHO cell line was initiated from a biopsy of an ovary of an adult Chinese hamster. The N-VERO cells have been employed extensively in virus replication studies and plaque assays. The hybridomas can be grown in suspension, while the CHOK1 and the N-VERO cells are anchorage-dependent cells.

2.1.2 Media

The insect cell lines (SF9 cells and TN368 cells) were sub-cultured in TNMFH (Sigma Chemical Co., MO) medium in stationary tissue culture flasks. However, these cells were also grown in a serum free medium (Hink, 1991) with lipids (SFML). The SFML medium was obtained from Dr. Fred Hink (Department of Entomology, The Ohio State University). The TNMFH medium contains 10% v/v fetal bovine serum (FBS), while the SFML medium is supplemented with lipids. The osmolarity and pH of these two media were 350-370 mOsm/L and 6.2 respectively. Ham F12 (Sigma Chemical Co., MO) medium containing serum (10% v/v) was used for the CHOK1 cells, and RPMI (Sigma Chemical Co., MO) medium containing serum (10%v/v) was used for the hybridomas. The N-VERO cells were subcultured in Medium 199 (M199) (Sigma Chemical Co., MO) supplemented with serum (10%v/v).

Experiments were also conducted with Hank’s balanced salt solution (HBSS). The composition of HBSS is shown in Table 2.1.
Table 2.1
Composition Of HBSS

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount in g/L</th>
</tr>
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<tbody>
<tr>
<td>NaHCO₃</td>
<td>0.35</td>
</tr>
<tr>
<td>KCl</td>
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</tr>
<tr>
<td>MgCl₂·6H₂O</td>
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</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.10</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
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</tr>
<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.186</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.06</td>
</tr>
<tr>
<td>Glucose</td>
<td>15.0</td>
</tr>
</tbody>
</table>
2.1.3 Protective Additives

Five different classes of additives were used in this study: (a) Pluronic F68 (BASF Corporation, NJ); (b) polyvinyl alcohol (PVA) (Aldrich Chemical Co., WIS); (c) polyethylene glycol (PEG) of molecular weights 400, 1000, and 4000 (J.T. Baker Chemical Co., NJ; Sigma Chemical Co., MO; and Amend Drug & Chemical Co., NJ respectively); (d) Methocels (Dow Chemical Co., Midland, MI) of grade E4M, and E50 and A4M; and (e) Dextran (Sigma Chemical Co., MO).

Pluronic is the trade name for a class of non-ionic surface-active agents that are poly(oxyethylene)-poly(oxypropylene) block copolymer surfactants, with a generic name of poloxamers (Attwood and Florence, 1983). The particular poloxamer used for the study is Pluronic F68. About 80% of the total molecular weight of Pluronic F68 is C$_2$H$_4$O- portion, and the remaining is C$_3$H$_6$O- portion. The Methocels are the trade names for methylcelluloses (Methocel A4M) and hydroxypropyl methylcelluloses (Methocel E4M and E50). The number immediately following the letter A or E corresponds to the viscosity in millipascal-seconds of that type of Methocel measured at 2% concentration in water at 20°C. The letter M represents 1000 when designating viscosity. These are water-soluble polymers derived from cellulose, which is a natural carbohydrate containing a basic repeating structure of anhydroglucose units. These compounds are widely used as emulsifiers, thickeners, binders, water retention agents, suspension aids, surfactants, and protective colloids. The chief difference between hydroxypropyl methylcellulose and methylcellulose is that methylcellulose is made using methyl chloride, whereas hydroxypropyl methylcellulose is made using methyl chloride and propylene oxide. Both Pluronic and the Methocels are not reactive in biochemical and enzymatic systems, and are stable. The molecular weights of all the protective additives and the concentrations of these additives used for the present study are indicated in Table 3.2.
2.2 Experimental Techniques

2.2.1 Interfacial Tension Measurements

As mentioned in Chapter 1, it has been proposed that the protective additives in the culture medium affect the interfacial properties between the different phases. The interfacial property that has been studied is the interfacial tension. In a typical bioreactor, three phases (cell, liquid, and vapor) are involved, giving rise to three corresponding interfacial tensions ($\gamma_{lv}$ - interfacial tension between liquid and vapor phases, commonly termed as the surface tension of the liquid medium, $\gamma_{cv}$ - interfacial tension between cell and vapor phases, and $\gamma_{cl}$ - interfacial tension between cell and liquid phases). Out of the above three, only $\gamma_{lv}$ can be determined experimentally. The values of $\gamma_{cv}$ and $\gamma_{cl}$ can be calculated from semi-empirical equations with experimentally determined values of $\gamma_{lv}$ and contact angle ($\theta$) that the liquid makes on the cell surface. With the available values of interfacial tensions, the change in free energy for the process of adhesion ($\Delta F^{adh}$) taking place between the cells and the air-medium interface can be determined. The magnitude of $\Delta F^{adh}$ determines whether the cell-gas adhesion will occur under a particular set of conditions.

Several techniques have been developed to obtain the values of $\gamma_{lv}$ and $\theta$ for various cell lines. For this present study, two different techniques have been used for determining $\gamma_{lv}$: (i) Maximum Bubble Pressure (MBP) method; and (ii) Wilhelmy Plate method. The value of $\theta$ has been determined by two methods: (i) Axisymmetric Drop Shape Analysis (ADSA) method, and (ii) Wilhelmy Plate method. A third method (Bubble Contact Angle method) for the determination of $\theta$ has also been suggested for the measurement of contact angle.
2.2.1.1 Surface Tension Measurements

The Maximum Bubble Pressure (MBP) method determines the dynamic surface tension, while the Wilhelmy Plate method determines the equilibrium surface tension of the liquid. In the case of MBP method (Adamson, 1990) bubbles of an inert gas (nitrogen) are slowly blown into the test liquid through tubes projecting below the surface of the liquid. Considering a hemispherical shape of the bubble, the radius of the bubble will be equal to the radius of the tube and, since the radius is at its minimum, $\Delta P$ is at its maximum. Equating the work done against this pressure difference to the decrease in surface free energy, one gets:

$$\Delta P 4\pi r^2 \, dr = 8\pi \gamma \, dr \quad \text{(2.1)},$$

and the above equation simplifies to

$$\Delta P = \frac{2\gamma}{r} \quad \text{(2.2)}.$$

Therefore, $\gamma$ can be determined from the knowledge of the pressure difference and the radius of the tube. This method is good to a few tenths percent accuracy. The values obtained by this method do not depend on the contact angle and this method will be able to measure only the surface tension value of the liquid. The influence of the impurities on the surface tension values is also minimized in this method.

The instrument used for measuring the $\gamma$ is a Sensadyne 6000 Surface Tensiometer from ChemDyne Research Corp. (Milwaukee, Wisconsin). A schematic diagram is shown in Figure 2.1.
Figure 2.1   A schematic diagram of Sensadyne 6000 Surface Tensiometer.
In this case, nitrogen is blown slowly through two probes of different radii ($R_1$ and $R_2$) that are immersed in a test fluid. The bubbling of the gas through the probes produces a differential pressure difference ($\Delta P$) which is sensed by a differential pressure transducer. The transducer output is conditioned and sent through an analog interface board to the computer where it is scaled and offset in relation to a previously computer-calculated calibration curve. The value of surface tension is measured each time a new surface is formed and the bubble is released from the orifice. Keeping the two probes at approximately the same immersion depth cancels the effects of liquid level. Prior to data collection, the instrument has to be calibrated with two liquids of known surface tension (water and ethanol).

This method can be termed as dynamic as freshly formed liquid-air interfaces are involved. Dynamic surface tension is defined as any non-equilibrium value of surface tension that arise when the surface of a solution is extended or contracted. As the surface moves towards equilibrium, either by adsorption or desorption of solute, the surface tension changes towards its static value. Therefore, the surface tension of the liquid is not established immediately on creation of the surface. To attain equilibrium, the molecules have to rearrange themselves in a preferred orientation at the created surface, with an increase in their intermolecular distance. An equilibrium surface tension can also be determined with the MBP method by using a minimum bubble flow rate such that the bubble surface and the liquid comes to an equilibrium in the interval between two bubbles. A usual bubble rate of one per second is used, however, the bubble rate can be adjusted to a lower value. In the case of a pure liquid, equilibrium values are easily reached (a few milliseconds) within a bubble rate of one bubble per second. However, when surface-active components are present in the liquid, more time is required to attain equilibrium, as it depends on the rate of diffusion of solute molecules to the surface. The rate of diffusion is dependent on the size of the molecule and its concentration (Ross and
Morrison, 1988). As the instrument can attain only a certain minimum bubble flow rate, it is incapable of measuring the equilibrium surface tension value in a number of cases where surface-active agents are present.

In the Wilhelmy plate method, a thin plate, such as a microscope cover glass or a platinum plate, support a meniscus whose weight can be given as (assuming zero contact angle)

\[ W_{\text{total}} = W_{\text{plate}} + P\gamma \]  

(2.3)

where P is the wetted perimeter. This particular technique is used in the DCA-322 system by CAHN Instruments Inc., Cerritos, CA. Figure 2.2 shows a schematic diagram of the instrument used.
Figure 2.2 A schematic diagram of Wilhelmy Plate method.
The forces acting on the plate in such an instrument for a non-reacting low viscosity liquid operating at low stage speed, can be written as (Giannotta et al., 1993)

\[ F = F_w + F_{ie} + F_b \]  \hspace{1cm} (2.4),

where \( F_w \) represents the contribution due to the weight of the plate, \( F_{ie} \) is the contribution of interfacial energetics, and \( F_b \) is the contribution due to buoyancy. Equation (2.4) can be written as:

\[ F = mg + \gamma \cos \theta + (x - x_0)Adg \]  \hspace{1cm} (2.5),

where \( m \) is the sample mass, \( g \) is the gravity constant, \( \gamma \) is the interfacial tension between the two contacting phases, \( P \) is the wetted perimeter of the plate, \( \theta \) is the contact angle as shown in Figure 2.2, \((x-x_0)\) is the length of the immersed portion of the plate, \( A \) is the cross-sectional area of the plate, and \( d \) is the density of the liquid. An additional term will be introduced when a viscous liquid is used or a viscoelastic fluid at higher stage speeds is used to account for the non-negligible contributions from shear stress \( (\tau_0) \) exerted by fluids on the solid surface. Equation (2.5) will become:

\[ F = mg + \gamma \cos \theta + (x - x_0)Adg + P(x - x_0)\tau_0 \]  \hspace{1cm} (2.6).

The method used here is simple, reliable and custom-made for use in characterizing the wetting behavior of fully hydrated surfaces. Both advancing and receding contact angles are obtained.

This instrument has also the potential for calculating the dispersive (non-polar) component and the polar component of the surface tension of a liquid. In order to fully
characterize a liquid for polar and non-polar components, a solid must be selected that will measure only dispersive forces and has a surface energy low enough to insure a non-zero contact angle (i.e., a non-polar solid). Teflon, for example, is suitable as it has high dispersive energy and low surface energy.

In order to achieve a correct value of \( \gamma_{lv} \) of a liquid, it is important to ensure that the contact angle made by the liquid on the slide is very close to zero. A deviation would give an incorrect result. One must also ensure that the slide used is clean such that there is no dust to influence the reading obtained for \( \gamma_{lv} \). Removal of dust particles from the platinum plate has been done by heating the plate over a flame until the plate becomes red hot. Roughening of the platinum plate is used to ensure that the contact angle of the thin plate in contact with the liquid is always close to zero. Roughness of a surface has the effect of making the contact angle different from the actual contact angle. If the contact angle on the smooth surface was less than 90°, roughness decreases the contact angle. Quantitatively, roughening is measured by the ratio of the real to the apparent surface, and this ratio \( r \) can be expressed as:

\[
r = \frac{\cos \theta}{\cos \theta^1}
\]  
(2.7)

where \( \theta \) is the angle made on the smooth surface and \( \theta^1 \) is the average angle made on the rough surface.

### 2.2.1.2 Contact Angle Measurements

The Axisymmetric Drop Shape Analysis (ADSA) method is based on the shape of static drop. Small drops tend to be spherical because surface forces depend on the area, which changes as the square of the linear dimension, whereas distortions due to gravitational effects depend on the volume, which changes as the cube of the linear
dimension. The shape of a sessile drop is a result of a balance between the gravitational effects and the surface tension effects. In the absence of gravity, the drop will be spherical, since this geometry encloses the maximum volume within a minimum surface area. Gravity forces acting on the drop will tend to lower its center of mass, thus, flattening it and increasing the surface area. In this case, the drop is formed and measurements made under conditions such that the drop is not subjected to disturbances. The pendant drop method can be used to measure the interfacial tension, whereas the sessile drop method can be used to measure both the contact angle and the interfacial tension. The main advantage of this method is that a small quantity of liquid can be used to obtain the data. The accuracy is usually several tenths of a percent and is also suitable for long-range changes in surface tension.

The ADSA method currently used in our laboratory was initiated by Rotenberg et al. (1982). The software developed (ADSA-P) determines both the surface tension and the contact angle from the profile of the drop. It involves a numerical scheme that is a combination of Least Squares Fit and the Newton-Raphson method, in conjunction with Incremental Loading method (Cheng et al., 1990). ADSA-P is suitable for both pendant drops and sessile drops. For the present study, we intend to measure the contact angle made by a liquid on the surface of a monolayer of cells deposited on a solid substrate. Since in most of the cases the contact angle is quite low, the accuracy of the results obtained with the ADSA-P program is not very high. A modification of the ADSA-P program (ADSA-CD program) was used by Duncan-Hewitt et al. (1989) to measure the contact angles on cell surfaces. This program is specifically suitable for systems with low contact angle, as in case of biological systems. ADSA-CD uses the average contact diameter, obtained from the top view of the system, to calculate the contact angle. The other inputs required for this software are the drop volume, liquid surface tension ($\gamma_{lv}$), and the difference in densities between the liquid and the vapor phases.
The ADSA-CD program relies on numerical integration of the Laplace equation of capillarity, which is (Hiemenz, 1986):

\[ \Delta P = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right) \]

where \( \gamma \) is the interfacial tension, \( R_1 \) and \( R_2 \) represent the two principal radii of curvature, and \( \Delta P \) is the pressure difference across the interface. When a sessile drop is placed on the solid surface, the liquid volume displaces the volume occupied by the gas at that location. The weight of the displaced gas volume exerts a buoyant force on the meniscus of the liquid drop, and so the density difference (\( \Delta \rho \)) between the gas and the liquid is required. The hydrostatic pressure exerted by the liquid is equal to \( \Delta \rho g z \), where \( g \) is the acceleration due to gravity and \( z \) is the vertical height of the drop measured from the reference plane. The value of "\( g \)" is required for the particular place in which the measurement is being made. The hydrostatic pressure (\( \Delta \rho g z \)) can be equated to the pressure due to surface tension to provide the value of \( \gamma \). The general form of the equation is (Hiemenz, 1986):

\[ \gamma \left( \sin \frac{\phi}{x} + \frac{1}{R_1} \right) = \frac{2γ}{b} + \Delta \rho g z \]

where \( b \) is the radius of curvature at the top of the drop, \( \phi \) is the angle made with the axis of symmetry by \( R_2 \) and \( x \) is equal to \( R_2 \sin \phi \). \( R_1 \) and \( R_2 \) are perpendicular to each other.

In this method an objective function is developed to obtain the deviation between the physically observed curve from a theoretical Laplacian curve, and then this deviation is minimized by using numerical techniques.
From the above equation it can be seen that one needs to know the difference in density between the liquid and the vapor, gravitational acceleration, and the coordinates of the profile of the drop along with the volume of the drop and $\gamma_{lv}$ of the test liquid. Figure 2.3 provides a scheme of the procedure followed for obtaining the contact angle values using the ADSA-CD technique.
Figure 2.3  A scheme of the procedure followed for obtaining the contact angle values using the ADSA-CD technique.
The profile of the drop can be obtained from a photograph of a drop or can be obtained directly with the use of a video camera. The photograph of the drop can be manually digitized to obtain the coordinates of the drop with respect to a reference plane. This process is time consuming and is also dependent on the efficiency of the operator. Therefore, instead of a photograph, a video camera can be used directly to obtain an analog video signal of the sessile drop. This signal is transmitted to an image processor where the analog signal is converted to a digital signal containing the image data as digital picture elements, or pixels. The digital pixel data are then stored in frame memory one frame at a time. A computer is used to acquire the image from the image processor and to perform the image analysis and computation. Each set of the image of the drop should be calibrated with an accurate grid that can also correct for any optical distortion. On comparing the pixel coordinates of the drop with that of the calibration grid, the pixel coordinates of the profile of the drop can be converted to Cartesian coordinates.

In order to acquire the drop profile coordinates, digital image processing has been employed. The camera used is a CCD video camera. The Pulnix (model No. TM-640) CCD camera allows us to film at a maximum speed of 120 Hz when operated at a non-interlaced mode. The camera also has a variable shutter which can be controlled from 1/63s to 1/15750s. It has a pixel resolution of 649H x 491V. A SVHS is available for recording the event when the video camera is used.

Digitizing is the process of translation of information from visual images (continuous) to computer images (discrete digits). The digitization process is performed by scanning over the visual image. The digitization process assigns an integer value, called the gray value, usually between 0 and 255, to every pixel, based on the light intensity of the image at each and every point. Depending on the recording media, the image is either directly, or through one extra step, grabbed using a Dipix P360 frame grabber, which allows users to perform image processing operations on a personal
computer (PC). Image processing is a process of enhancement of digitized images to improve the images so that the final image analysis steps can be handled more easily. Many of the image processing operations can be performed by using the library functions available from the Dipix library written in Turbo C. The 486 PC is equipped with a SVGA card, and has a maximum resolution of 800 x 600 with 256 colors.

There are some problems associated with the measurement of the contact angle on the biological surfaces: (i) low contact angles reduces the accuracy of the system; (ii) contact angle decreases as the liquid is adsorbed by the biological surface; and (iii) the biological surface is heterogeneous in nature, which distorts the shape of the sessile drop on the cell surface. Moreover, it should be kept in mind that the cells should be maintained in fully hydrated state and the liquid used to form the sessile drop should be physically and biologically compatible. There is another important consideration - surface-active substances such as proteins can be excreted from the cells and the adsorption of these secreted compounds may change the surface tension of the drop of the test liquid.

In order to obtain a monolayer of cells, slides from the culture chambers can be used directly in the measurement. After the cells have become confluent, they are allowed to dry for a certain period of time, usually 12 to 15 minutes (van Oss et al., 1975), before the actual measurement of the contact angle. In the case of the ADSA-CD technique, the pixel coordinates can be picked manually, and these points can be used to calculate the average diameter of the drop. A precisely manufactured grid has been used for the calibration of the image. Figure 4.4 gives a schematic diagram of the experimental apparatus used for the study.

Care should also be taken regarding the proper alignment of the camera and the sample. This can be done with a plumb line. The accuracy of the experimental data is also dependent on several experimental conditions, such as focusing of the camera,
lighting, the effect of optical distortion, and the errors in the measurement of local gravitational acceleration, volume of the drop, and the density difference. Precautions should be taken to minimize evaporation and also to isolate the system from any external disturbances, such as air vibration.

The second method used for measuring the contact angle is the Wilhelmy Plate method. The $\gamma_V$ of the liquid has to be determined first, and once this value is known, it can be used to measure the contact angle made by the liquid medium on a monolayer of cells. For this purpose, cells are grown on both sides of the slide, and DCA-322 is used to measure the contact angle with a liquid of known $\gamma_V$. The procedure followed is same as that used for the determination of $\gamma_V$. Care should be taken to ensure that the slides are confluent with cells during the experiment. For that purpose, only anchorage-dependent cells are used for these measurements. The advantage of this method is that the cells are in contact with the liquid medium and therefore, they are always maintained in a hydrated condition.

In experimental measurements, it is frequently difficult to obtain reproducible contact angle values. In particular, the angle measured when the solid surface is advancing into the liquid may be greater than when the plate is being withdrawn. This hysteresis of contact angle is due to the difference between the advancing and the receding angles. The large advancing contact angle may be due to a film of some material that prevents the liquid from adhering to the solid. After contact with the liquid, this film may be wholly or partially removed, so that the contact between the liquid and the solid becomes more complete, giving a smaller receding angle.

A third method is recommended for the measurement of the contact angle between the liquid and the cell monolayer. The bubble contact angle (BCA) method is used for measuring the contact angle made by a bubble on the cell surface in the presence of the liquid. This might be an improvement on the other processes as it approximates
the conditions existing in the bioreactor more closely. The cells can be tested without pre-drying and denaturation, which means they remain in their native hydrated form. However, the interfacial tension of the liquid has to be measured by a different method, like the MBP method. One should also be careful so that the cells forming the monolayer on the glass slide do not become detached from the surface during the experiments. Reliable results can be obtained with anchorage-dependent cells.

The contact angles of air bubbles on the test slide can be measured by injecting an air bubble from a micro-syringe into a water-tight chamber containing the test liquid. The bubble, introduced at the bottom of the chamber, floats to the top of the chamber and comes to rest at the test slide, which is placed horizontally at the top of the liquid. The contact angle can be measured directly with a microscope fitted with a goniometer eyepiece. The profile of the bubble can also be analyzed using image-processing techniques. Software exists that can calculate the interfacial tension and the contact angle made by the bubble on a surface from the coordinates of the profile of the bubble. However, the program needs to be updated.

2.2.2 Bubble Rupture Experiments

The shear-sensitivity of the cells can be tested by determining the number of cells damaged when single bubbles are ruptured in a volume containing a particular cell suspension. The experimental setup is similar to that used by Trinh et al. (1994). A schematic diagram is shown in Figure 2.4.
Figure 2.4 A schematic diagram of the apparatus used for the bubble rupture experiment.
A particular volume of cell suspension was taken in a glass column of capacity 1-2 mL. An automobile coil connected to a power source is used to generate sparks. Bubbles can be introduced in the glass column with a hypodermic needle. As the bubbles reach the top of the liquid column, sparks were used to rupture these bubbles. For this experiment, a certain number of bubbles were ruptured in order to estimate the degree of cell damage due to a single bubble rupture. The number of cells killed per bubble ruptured can be estimated from the change in total and viable cell counts. The cell counts were done using the trypan blue exclusion test with a hemacytometer. The experiments were conducted with cell suspensions containing various protective additives. Cell suspension without any protective additives was used as a control. Different types of protective additives were used for this experiment to estimate the relative protection provided by the different medium additives with respect to bubble rupture.

2.2.3 Visualization Studies

The phenomenon of cell-gas adhesion was observed through a microscopic lens using the experimental apparatus shown in Figure 3.1. The glass column contains the cell suspension and the hypodermic needle can introduce bubbles in the cell suspension. As the bubble rises through the liquid, cells tend to adhere to the bubble surface. The bubble surface can be studied by using the image recording equipment which consisted of a CCD video camera (Pulnix, model no. TM-640), an Infinity CFM lens, and a super VHS video recorder. A suitable light source was used such that the illumination allowed the cells to appear as white spots. The age of the bubbles ranged between 15 to 30 seconds on the solution surface. The experiments were conducted with different protective additives and cell suspension without any protective additive was used as a control. The purpose of the experiment was to study the effect of the presence of protective additives on the degree of cell-gas adhesion.
2.2.4 Lipid Analysis By Thin-Layer Chromatography

2.2.4.1 Extraction Of Lipids

The plasma membrane separates the contents of a cell from its environment and plays an important role in the life and viability of the cell. The first step in any lipid analysis of cell is the isolation of lipids from tissues by extraction with organic solvents, and the removal of non-lipid contaminants from this extract. Lipids are bound to one another or to the proteins by four types of forces: (i) van der Waals' interactions; (ii) hydrogen bonding; (iii) electrostatic bonding; and (iv) covalent bonding. Solvent extraction used to extract lipids break all these bonds, except the covalent bonds. Therefore, the lipids that are covalently bound to the polypeptides or polysaccharides moieties will not be extracted at all by the organic solvents and would remain in the so-called "non-lipid" residue. The four steps involved in the lipid extraction from tissues are:

(i) Homogenization of the tissue in the presence of solvent;
(ii) Separation of the aqueous and organic phases;
(iii) Removal of non-lipid contaminants;
(iv) Drying of the extract and removal of solvent.

Lipids can be extracted from tissues by a number of organic solvents, but special precautions are necessary to ensure that enzymes are deactivated and that the recovery is complete. The ideal solvent or solvent mixture for extraction of lipids should be sufficiently polar to remove all lipids from their association with cell components, but should not react chemically with these lipids. At the same time, the polarity of the solvents should not be very high so that triacylglycerols and other non-polar simple lipids do not dissolve. The solvents should also prevent enzymatic hydrolysis of lipids. The solvents usually used for extraction are petroleum ether, diethyl ether, chloroform, methanol, ethanol, isopropyl alcohol and acetone, since the lipids are soluble in organic
solvents. It is believed that no single pure solvent is suitable as a general purpose lipid extractant. Phospholipids, glycolipids, and sterols of tissues are associated with proteins; and because water is involved in the bonding between the protein and the lipid, a dehydrating solvent, such as ethanol or methanol, must be used to rupture the lipid-protein linkage. Acetone is a poor solvent for phospholipids, however, glycolipids are soluble in acetone. The most common solvent system used for extraction of lipids are chloroform and methanol.

The most popular method for extraction is the Folch method (Folch and Lees. 1957; Kates, 1986). This extraction gives 95 to 99 percent recovery of lipids but gangliosides and occasionally some of the glycolipids and more polar acidic phospholipids may be lost during the washing step. Lysophospholipids are only partly recovered. This method gives excellent recoveries for neutral lipids, diacylglycerophospholipids and most of the sphingolipids.

The cell suspensions were collected in centrifuge tubes and centrifuged at 2000 rpm for 5 minutes to form a pellet. The pellet was resuspended in 1 mL of HBSS. In this way, the lipids present in the medium was excluded and the results obtained on the TLC plate would show only the lipids present in the cells. The suspension was transferred to a glass-stoppered centrifuge tube, and 3.75 mL of methanol-chloroform (2:1 v/v) mixture was added. The sample and solvent were homogenized by vortexing and left at room temperature for 1-2 hour with intermittent shaking. The mixture was then centrifuged, and the supernatant, containing the lipids from the cells was collected in another glass centrifuge tube with Pasteur pipette. The solid residue was re-suspended in 4.75 mL of methanol-chloroform-water mixture (2:1:0.8 v/v) and homogenized before centrifuging. Further washing with the solvent was conducted to extract the remaining lipids from the cells. The supernatants were combined and 2.5 mL of chloroform and 2.5 mL of water was added to it. This resulted in the formation of a two-phase system: the lower phase
containing mainly chloroform, while the upper phase containing mainly water. The resultant mixture was further centrifuged for separating the different phases. The lower layer containing the lipids was withdrawn and can be diluted further with benzene. The non-lipid contaminants will be in the upper phase. However, this polar upper phase may also contain some of the polar lipids, like lysophospholipids, gangliosides and others. Removal of the solvent is usually done by evaporation of the lipid extract under reduced pressure or by nitrogen blow-down. However, care should be taken so as not to evaporate the extract to complete dryness, as the lipids are unstable in the dry state. The mixture was evaporated and the residue was dissolved in a known volume of chloroform-methanol solution (1:1 v/v). There are other more extensive, and therefore more time-consuming, extraction procedures that will guarantee very little loss.

Most of the lipids are typically surface-active and thus they form stable emulsions. Usually the best way of breaking the emulsions is centrifugation, which mostly results in complete phase separation. For larger amounts of lipid extract, a small amount of benzene facilitates phase separation. The major merits of Folch's method are that much smaller amounts of solvents are required and that less non-lipid material is retained in the extract.

The organic solvents may also dissolve non-lipid contaminants, like sugars, urea, amino acids and salts. The amount of non-lipid contaminants is significant in the presence of polar solvents. The non-lipid contaminants are removed from the solvent by evaporation, and then dissolving the remaining lipids in non-polar solvent system. The non-polar solvents dissolve only lipids and leave many of the extraneous non-lipid substances behind. However, this kind of separation is not very complete, though this is simple to use when there are a number of samples, as it is less time-consuming.

Purified lipid extracts deteriorate rapidly on storage. The lipid extracts should be stored at very low temperatures to prevent decomposition. It has been found that
polyunsaturated fatty acids will auto-oxidize very rapidly if left unprotected in air. Although natural tissue antioxidants may afford some protection, an additional amount of antioxidant, such as BHT (butylated hydroxy toluene), can be added.

2.2.4.2 Analysis Of The Lipid Extract

An analysis of the lipid composition profile from a given source would involve separation of the lipids in different categories, followed by their identification on comparison with authentic standards. Among the different methods used for separation of a lipid mixture, the most important is chromatography. Chromatography has been widely used in separation of lipid mixtures, and the increasing amounts of information available on lipids is mainly dependent on the knowledge of chromatography. Chromatography has been defined as the physical method of separation in which the components to be separated are distributed between two phases, one of which is a stationary bed of large surface area, and the other is a fluid that percolates through the stationary bed. The stationary solid phase may be a solid having, for example, adsorptive, ion-exchange, or complex-forming properties.

Lipid samples obtained from the tissues are usually complex mixtures of individual lipid classes. In order to separate these mixtures into separate classes, different chromatographic techniques are usually employed. Adsorption chromatography is the most popular method used. Adsorption chromatography is based on the differences in the degree to which lipid components are adsorbed on to a solid support, relative to their solubility in an appropriate solvent. Lipids are held by the adsorbents in a variety of ways, including hydrogen bonding, van der Waals' forces and ionic bonding. The polarity of the functional group in the lipid dictates the strength of the binding of the lipid to the adsorbent. Lipids are released from the adsorbent by passing solvents of increasing polarity through it, and are therefore separated according to the number of fatty acids in
the molecule and the number and type of other polar functional groups. In case of thin-layer chromatography (TLC), which is one of the adsorption chromatographic methods, the adsorbents are supported as thin layers on glass plates and the eluting solvent is allowed to pass through the adsorbent by capillary action.

Our objective was to separate the lipids present in the samples in broad categories, and for this purpose the method used was thin layer chromatography (TLC). TLC is usually the method used for analysis of simple lipids and it may be performed on the microgram to 100 mg scale. The apparatus used for this purpose is inexpensive and also the results are obtained in a short period of time. Besides being rapid, TLC is also sensitive and gives better resolution than column chromatography. In order to bring about an effective separation by TLC several variables should be taken into consideration, such as physical properties of the components in the lipid mixtures to be separated, of the adsorbent layers, and of the solvent systems.

A very fine grade of silica gel, which is a partially hydrated silicon dioxide, is by far the most common adsorbent used for TLC and this may contain calcium sulfate as a binder to ensure adhesion of the layer to the plate. Silica gel is the most commonly used adsorbent for separating classes of lipids based on number and nature of the various polar functional groups (like, ester bonds, phosphate, hydroxyl, and amine groups) in the molecules. The adsorptive property of the silica gel is mainly due to the hydroxyl groups, which are attached to the surface and can be free or hydrogen-bonded. In addition, there is the water of hydration, which exists first in a strongly bound layer and then in one or more loosely bound layers on the surface. The loosely bound water can have a marked effect on the reproducibility of separations, especially of non-polar lipids, as it can be readily removed inadvertently. So to ensure reproducibility in retention times and resolution, it is better to arrange that only the strongly bound water layer is permitted to remain. For analytical purposes, layers of adsorbent 0.25 mm thick or less give
maximum resolution. The resolution decreases with the thickness of the layer.

Samples are applied as discrete spots or as narrow streaks, 1.5 - 2 cm from the bottom of the plate, in a solvent (frequently chloroform) by means of an applicator, and the plate is then placed in a tank containing the eluting solvent. The solvent moves up the plate by capillary action taking the various components with it at differing rates, according to the extent to which they are held by the adsorbent. As little as 0.5 mg of lipid can be applied as a spot to a TLC plate. When the solvent reaches the top of the plate, the plate is removed from the tank, dried, and sprayed with a reagent that renders the lipids visible. The spray may be a chemical agent, which is specific for certain types of lipid, or for certain functional groups, or it may be a non-specific reagent that renders all lipids visible. Charring followed by photodensitometry is probably the most popular method of quantifying components separated by TLC. In this case, the plates are sprayed with 30% aqueous sulfuric acid or 10% methanolic sulfuric acid and heated on a hot plate at 180°C for 5 to 20 minutes. As a result of heating, all lipids (and any other organic compounds) will appear as black spots.

There are three common methods for the detection of compounds on thin-layer plates: (i) detecting agents may be used after development of the chromatogram; (ii) detecting agents may be used before development of the chromatogram; (iii) radioactivity of isotopically labeled mixtures may be utilized to locate the positions of the zones. In some cases, lipids may be identified by their color or by their fluorescence after UV excitation. The technique of spraying reagents on the layer after development is most commonly used.

Choice of the eluting solvents in case of the TLC is very important in obtaining the final accurate result. A variety of solvents are available that can be used in one-direction TLC. In determining the resolution of the TLC column, a quantity known as the Rf value has to be determined. Rf value is defined as the ratio of the distance moved by a
component, as measured from the origin of spotting to the center of the spot, to the
distance traveled by the solvent front. The Rf value should lie between 0.3 to 0.8. The
Rf value is controlled by choice of suitable solvents. The solvents mentioned below are
given in order of their increasing polarity: petroleum ether < cyclohexane < carbon
tetrachloride < trichloroethylene < toluene < benzene < dichloromethane < chloroform <
diethyl ether < ethyl acetate < acetone < n-propanol < ethanol < methanol < water. In our
case, when the lipids from the cells are to be analyzed, different solvents are to be used to
obtain the results. Jenson and Pitas (1976) also have provided a list of different solvents
that are commonly used in TLC. Lipids must be dissolved in a relatively non-polar
solvent. Polar solvents will cause local deactivation of the adsorbent, allowing the
applied spot of lipid to spread widely; and this will result in poor resolution. Neutral
lipids such as acylglycerols and esters may be dissolved in petroleum ether, but polar
lipids, such as phospholipids, will require chloroform or chloroform-methanol mixtures.

Chromatograms may be developed by ascending, descending, or radial flow of the
solvent. Development can be carried out discontinuously, using one pass or multiple
passes of the same solvent; in a step-wise fashion with different solvents; with a
continuously changing solvent composition gradient; or by the so-called continuous
process, in which the solvent or the compounds are removed when they arrive at the
distant end of the TLC plate. Complicated lipid mixtures cannot always be separated by
one-directional TLC, but can often be resolved by rechromatography in a second
direction. Considering the complex system present in the cell membrane, application of
two-dimensional chromatography is more appropriate. In this method, the sample is
applied to the plate as a spot in the bottom left-hand corner of a square TLC plate and the
plate run normally in a selected solvent system. When the solvent has run close to the top
of the plate, the plate is removed and dried, taking care so as not to deactivate the
adsorbent. The plate is then turned anti-clockwise through 90° and redeveloped with a
second solvent system.

2.2.4.3 Precautions Taken

In order to avoid sample contamination, clean glassware was used to handle the samples prior to chromatography. Storage and handling of samples were done in glass containers covered with aluminum foil or teflon-lined lids. As required, the glassware was first cleaned with acid or a suitable detergent, then by chloroform-methanol (2:1 v/v) prior to use. No plastic or metal containers were used. All solvents used were at least of reagent or chromatographic grade purity. To determine the reliability of a chromatographic analysis on a biological sample, replicate experiments (at least two or more replicate) were carried out with different representative samples. For each experiment, at least two chromatographic runs were made.

Concentration of solutions was done by evaporation. Care was taken to concentrate the sample without degrading or losing the compound of interest. As the volume of sample was small, simple nitrogen blow-down procedure was used. The sample was taken in a small vial or tube, and a pasteur pipette attached to a rubber tubing to a nitrogen tank was inserted in the vial. The bubbling of the nitrogen gas evaporated the solution. After the solution was evaporated, the residue was sometimes reconstituted in a known volume of an appropriate solvent.
CHAPTER III

THE PROTECTIVE EFFECT OF SPECIFIC MEDIUM ADDITIVES WITH RESPECT TO BUBBLE RUPTURE

The content of this chapter has been accepted for publication in Biotechnology and Bioengineering.

3.1 Summary

A significant degree of cell damage is observed during suspension cell culture with air sparging. Protective agents can be added to the culture medium to protect the cells from damage. It has been observed that cells tend to adhere to air-medium interfaces and cell damage is mainly due to this cell-bubble interaction and protective additives have been found to prevent this cell adhesion to the bubble surfaces. In this paper, it is demonstrated that the interfacial tension between the air and medium is related to the effectiveness of the protective additives to prevent adhesion of cells to this interface. Five different types of additives (Pluronic F68, Methocels, Dextran, Polyvinyl alcohol, and Polyethylene glycols) were studied in an effort to determine their protective characteristics. Liquid-vapor interfacial tensions of the culture medium, with and without the additives, were measured by two different techniques (Maximum Bubble Pressure method, and Wilhelmy Plate method). In addition, visualization techniques showed that in the presence of certain protective additives cells do not adhere to the bubble surface. Results obtained from these experiments indicate that the additives which rapidly lower the liquid-vapor interfacial tension of the culture medium also prevent adhesion of cells to the bubble surface. Experiments have also been conducted to determine the number of
cells killed due to bubble rupture and it was observed that this number is related to the amount of cells adhering to the bubble surface.

3.2 Introduction

In large-scale suspension cell culture, one method of supplying sufficient oxygen to the culture is by air sparging; however, in many cases, significant cell damage is observed. It has been reported that most of this cell damage is associated with cell-bubble interactions (Bavarian et al., 1991; Chalmers et al., 1991; Garcia-Briones et al., 1992; Handa et al., 1987; Handa-Corrigan et al., 1989; Jobses et al., 1990; Kunas et al., 1990; Murhammer et al., 1990; Tramper et al., 1986), and recently this damage has been quantified with respect to bubble rupture at the air-medium interface (Trinh et al., 1994). This damage is further associated with the adhesion of cells to gas bubbles and bubble films (Bavarian et al., 1991, Chalmers et al., 1991, Garcia-Briones et al., 1992; Trinh et al., 1994). Therefore, in order to prevent cell damage due to sparging or mixing, cell adhesion to the air-medium interface should be prevented.

3.2.1 Protective Additives

It has been found over the years that certain polymeric substances can be added to the growth medium to protect suspended cells. A number of compounds have been studied with respect to their ability to protect cells: pluronics, hydroxyethyl starch, derivatives of cellulose, serum, tryptose phosphate, polyvinyl alcohol, bovine serum albumin, polyethylene glycols, and dextran. Table 3.1 gives a partial list of the various additives that have been studied as protective additives in the presence of air sparging.
Table 3.1

Protective Additives Used In Cell Culture With Air Sparging

<table>
<thead>
<tr>
<th>Protective additives</th>
<th>Cell types</th>
<th>Conc., (wt%)</th>
<th>Methods of cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluronic F68</td>
<td>LS Mouse cells</td>
<td>0.02%</td>
<td>Sparged, stirred (Kilburn et al., 1968)</td>
</tr>
<tr>
<td></td>
<td>Human Lymphoblastoid</td>
<td>0.05%</td>
<td>Aerated, agitated (Mizrahi, 1984)</td>
</tr>
<tr>
<td></td>
<td>NS1 myeloma, Hybridoma, BHK21, Lymphoblastoid</td>
<td>0.1%</td>
<td>Bubble column (Handa-Corrigan, 1987, 1989)</td>
</tr>
<tr>
<td></td>
<td>Hybridoma (CRL-8018)</td>
<td>0.1%</td>
<td>Sparged, agitated (Michaels et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>SF9</td>
<td>0.1%</td>
<td>Airlift (Maiorella et al., 1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2%</td>
<td>Airlift, sparged, agitated (Murhammer et al., 1988)</td>
</tr>
<tr>
<td>Methylcellulose</td>
<td>TN-368</td>
<td>0.3%</td>
<td>Sparged (Hink et al., 1979)</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>BHK-21</td>
<td>2.4%</td>
<td>Sparged, agitated (Telling et al., 1965)</td>
</tr>
<tr>
<td></td>
<td>Human Lymphoblastoid</td>
<td>0.1%</td>
<td>Aerated, agitated (Mizrahi, 1984)</td>
</tr>
<tr>
<td></td>
<td><em>Dunaliella</em></td>
<td>0.1%</td>
<td>Roux bottle, miniloop reactor (Silva et al., 1987)</td>
</tr>
<tr>
<td>HES</td>
<td>Human Lymphoblastoid</td>
<td>0.2%</td>
<td>Aerated, agitated (Mizrahi, 1984)</td>
</tr>
<tr>
<td>PEG</td>
<td>Hybridoma (CRL-8018)</td>
<td>0.1%</td>
<td>Sparged, agitated (Michaels et al., 1991)</td>
</tr>
</tbody>
</table>

(contd.)
Table 3.1 (Contd.)

Protective Additives Used In Cell Culture With Air Sparging

<table>
<thead>
<tr>
<th>Protective additives</th>
<th>Cell types</th>
<th>Conc., (wt%)</th>
<th>Methods of cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPB</td>
<td>BHK-21</td>
<td>6.0%</td>
<td>Sparged, Agitated (Telling et al., 1965)</td>
</tr>
<tr>
<td>PVA</td>
<td>Hybridoma (CRL-8018)</td>
<td>0.2%</td>
<td>Sparged, agitated (Michaels et al., 1991)</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>Hybridoma</td>
<td>1000 mg/l</td>
<td>Airlift loop reactor (Hulscher et al., 1988)</td>
</tr>
<tr>
<td>Serum</td>
<td>LS Mouse cells</td>
<td>10%</td>
<td>Sparged, stirred (Kilburn et al., 1968)</td>
</tr>
<tr>
<td></td>
<td>NS1 myeloma, Hybridoma, BHK21, Lymphoblastoid</td>
<td>10%</td>
<td>Bubble Column (Handa-Corrigan et al., 1987, 1989)</td>
</tr>
<tr>
<td></td>
<td>SF - 9</td>
<td>10%</td>
<td>Airlift (Maiorella et al., 1988)</td>
</tr>
<tr>
<td></td>
<td>Hybridoma (CRL 8018)</td>
<td>10%</td>
<td>Sparged, agitated (Kunas et al., 1990)</td>
</tr>
</tbody>
</table>
Presence of some of these additives also reduce cell-gas adhesion. In the presence of the protective agent Pluronic F68 cell-gas adhesion is absent (Garcia-Briones et al., 1992) and, as has been extensively reported (Clarke et al., 1992; Goldblum et al., 1990; HANDa-Corrigan et al., 1987, 1989; Jobses et al., 1990; Maiorella et al., 1988; Michaels et al., 1991, Murhammer et al., 1988; Murhammer et al., 1990; Papoutsakis, 1991, Smith et al., 1987, Trinh et al., 1994), is very effective in preventing cell damage.

3.2.2 Cell-Gas Adhesion

In 1970, Parker and Barsom reported that the chemical composition of the surface microlayer, which is the thin layer of liquid at the air-liquid interface, is very different from that of the subsurface water. A large amount of hydrophobic substances, especially microorganisms, tend to accumulate in this layer. This phenomenon of bacterial cell adhesion to the air-liquid interface has been studied widely (Blanchard et al., 1982; Dahlback et al., 1981; Hermansson et al., 1982; Hermansson et al., 1983; Kjelleberg et al., 1980; Kjelleberg, 1985). The contributing factors for cell-gas adhesion are various, including electrostatic forces, Van der Waals' forces, chemical interactions between the cell surface and the air-liquid interface, and hydrophobic interactions. Accumulation of amphiphilic substances at the air-liquid interface is mainly attributed to the tendency of the non-polar groups to avoid water, leading to the aggregation of the hydrophobic groups. Studies by Dahlback et al. (1981) have shown that the accumulation of bacteria on the air-liquid interface is directly proportional to the hydrophobicity of the bacterial cell surface. Since hydrophilic-hydrophobic and electrostatic interactions are responsible for the attachment of bacteria to the air-liquid interface, bacteria-gas adhesion is considered non-specific in nature (Kjelleberg, 1985). In the work reported in this paper, it is assumed that the adhesion of cells to the air-medium interface is non-specific in nature, and the main factor responsible for cell adhesion to the air-medium interface is
hydrophobic-hydrophilic interactions.

Bacterial cell-adhesion to a solid substrate is also non-specific, as supported by the experiments conducted by Absolom et al., (1983). They studied the phenomenon of bacterial cell adhesion to various polymeric surfaces, and proposed a relationship based on thermodynamic considerations, to predict the possibility of the cell-solid adhesion. According to Absolom et al., (1983), adhesion of the cells to the solid surface are dependent on the interfacial tensions ($\gamma$) between the three interacting phases: the solid (s), the bacteria (b) and the surrounding liquid (l). The possibility of cell adhesion to the solid surface is determined by the change in free energy for the process of bacterial cell adhesion ($\Delta F^{\text{adh}}$) to the solid substrate, and the thermodynamic relationship that determines the change in the free energy of the process is given by:

$$
\Delta F^{\text{adh}} = \gamma_{bs} - \gamma_{sl} - \gamma_{bl} 
$$

(3.1)

A negative value of $\Delta F^{\text{adh}}$ indicates that the process of adhesion is thermodynamically favorable while a positive value indicates that the process is unfavorable. Various experiments were performed to show the validity of the proposed thermodynamic relation (Absolom et al., 1983).

3.3 Proposed Mechanisms Of Protection By Additives

Several different mechanisms have been proposed to explain the protective action of the additives in the growth medium during cell culture (Michaels et al., 1991; Papoutsakis, 1991). The three most commonly held views are: (i) the additives have a nutritional effect on the cells; (ii) the additives interact with the cell membranes and strengthen them; and (iii) the protective action of the additives is entirely physical in nature and has no effect on the cell membrane.
Serum, which is added to the culture medium to enhance cell growth and acts mainly as a nutrient, was also found to act as a protective agent (Ramirez et al., 1990). The protection mechanism resulting from the nutritional effect is not significant for most of the other protective additives, as it is highly unlikely for the cells to metabolize high molecular weight polymeric substances, as shown by Mizrahi (1984).

The second mechanism of protection, the strengthening of cell membranes due to interaction with protective additives, has been shown by a number of researchers to have some validity. There exists several reports of direct interactions of additives, such as dextran and Pluronics, with cell membranes (Clarke et al., 1992; Cudd et al., 1989; Murhammer et al., 1990; Smith et al., 1987). However, only Goldblum et al. (1990) reported that these additives protect cells from well-defined shear stress in viscometric studies. According to Goldblum et al., (1990), there occurs active adsorption of the additives on to the cell membrane to form a protective layer. In contrast to Goldblum et al. (1990), Michaels et al. (1991) reported that Pluronic F68 and PEG "did not make the cells more shear tolerant" upon either short or long exposure to the additives. Additional evidence of interaction of Pluronic F68 with the cell membrane was provided by Zhang et al. (1992). By determining the mean bursting membrane tension and elastic area compressibility modulus with and without Pluronic F68 present, it was concluded that the addition of Pluronic F68 does strengthens cells.

Handa-Corrigan et al. (1989) presented another mechanism to explain the protective effects of the additives which is purely physical in nature. They suggested that cell damage is either due to shearing during liquid drainage from the bubble film, or is due to oscillatory disturbances caused by the bursting bubbles. They also suggested that the presence of Pluronic F68 forms a stable foam and thus protects the cells from the damaging shearing effects of film drainage and/or bursting bubbles.
Michaels et al. (1991) studied the effect of serum, Pluronic F68 and polyethylene glycol (PEG) on the growth of Hybridoma CRL-8018 cells. Their experimental findings corroborated the theory proposed by Handa-Corrigan et al. (1989) and did not support the concept of a biological or nutritional effect. However, to add further complexity to this question, Murhammer et al. (1988, 1990) reported that the protective action of Pluronic F68 was evident even in the absence of foam, inferring that stabilization of the foam layer, as proposed by Handa-Corrigan et al. (1989), is not the only reason for the protective action.

We propose another mechanism of protection that would explain some, if not all, of the actions of protective agents. Cell damage, as a result of bubble rupture at a gas-liquid interface is prevented by the inhibition of cell adhesion to these interfaces. This relationship was quantified recently by Trinh et al. (1994). It is further proposed that this prevention of adhesion is the result of changes in interfacial properties. In this paper, we will investigate the relationship between cell adhesion to gas-liquid interfaces, gas-liquid interfacial tension, $\gamma_{lv}$, and cell damage as a result of bubble rupture for specific medium additives.

3.4 Materials Used

The cell-line used in this study was Spodoptera frugiperda (SF9) insect cells. The cell-line was sub-cultured in TNMFH medium in stationary tissue culture flasks, however, these cells were also grown in a serum free medium with lipids (SFM-L). Interfacial tensions were determined for TNMFH, SFM-L, and Hanks' balanced salt solution (HBSS). Five different classes of additives were used in this study: (a) Pluronic F68; (b) polyvinyl alcohol (PVA); (c) polyethylene glycol (PEG) of molecular weights 400, 1000, and 4000; (d) Methocels of grade E4M, and E50; and (e) dextran. Details about these additives are summarized in Table 3.2.
Table 3.2
Description Of Protective Additives

<table>
<thead>
<tr>
<th>Additives</th>
<th>Molecular Weights</th>
<th>Concentration, % w/v</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 400</td>
<td>400</td>
<td>0.1</td>
<td>J.T. Baker Chemical Co., NJ</td>
</tr>
<tr>
<td>PEG 1000</td>
<td>1000</td>
<td>0.1</td>
<td>Sigma Chemical Co., MO</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>4000</td>
<td>0.1</td>
<td>Amend Drug &amp; Chemical Co., NJ</td>
</tr>
<tr>
<td>Pluronic F68</td>
<td>8400 (Goldblum et al., 1990)</td>
<td>0.1</td>
<td>BASF Corp., NJ</td>
</tr>
<tr>
<td>PVA</td>
<td>77 - 79 K</td>
<td>0.1</td>
<td>Aldrich Chemical Co., WIS</td>
</tr>
<tr>
<td>Methocel E50</td>
<td>65-80 K (Goldblum et al., 1990)</td>
<td>0.3</td>
<td>The DOW Chemical Co., MI</td>
</tr>
<tr>
<td>Methocel E4M</td>
<td>300-500 K (Goldblum et al., 1990)</td>
<td>0.3</td>
<td>The DOW Chemical Co., MI</td>
</tr>
<tr>
<td>Dextran</td>
<td>79 K</td>
<td>0.5 &amp; 5.0</td>
<td>Sigma Chemical Co., MO</td>
</tr>
</tbody>
</table>
3.5 Experimental Techniques

Four types of experiments were conducted to determine the relationship between additives and cell damage. Two of them: the Maximum Bubble Pressure method (Adamson, 1990; Bikerman, 1970) and the Wilhelmy Plate method (Adamson, 1990, Giannotta et al., 1993) were used to determine the interfacial tension, $\gamma_w$, of the culture medium, with and without protective additives. The third type of experiment involved visualization techniques in which the adhesion of cells to the air-medium interface as a function of medium additive was studied. The final technique involved quantifying the number of cells killed per bubble rupture as a function of medium additive.

3.5.1 Maximum Bubble Pressure Method

The dynamic liquid-vapor interfacial tension, $\gamma_{lv}$, was measured with a Sensadyne 6000 Surface Tensiometer from ChemDyne Research Corp. (Milwaukee, Wisconsin). This instrument uses the Maximum Bubble Pressure method (Adamson, 1990; Bikerman, 1970). This method gives the dynamic value of liquid-vapor interfacial tension as freshly formed liquid-vapor interfaces are involved. The typical bubble generation rate used was one bubble per second. This instrument operates by supplying a constant gas flow rate to the bubbler; since the maximum bubble size is a function of interfacial tension, the bubble rate also varies slightly as a function of $\gamma_{lv}$. For these experiments, the time between initial bubble formation and attainment of maximum bubble pressure was approximately 0.5 - 1.0 seconds, consequently, measured values of $\gamma_{lv}$ represent the dynamic interfacial tension for the surface "age" of approximately 1 second. Several experiments were performed at different gas flow rates and deviations in $\gamma_{lv}$ due to the changes of bubble rate in the range of 0.5 - 2.0 seconds were found to be negligible for all additives tested.
The advantage of this method is that the influence of impurities on the surface tension values is minimized and the interfacial tension of an interface unaffected by denatured proteins, RNA and DNA is determined. This is opposed to the method described next in which the interfacial tension of an "old" interface, presumably at equilibrium, is involved. Prior to data collection, the instrument was calibrated with two liquids, of known surface tension, as directed by the manufacturer. A more complete description of the principles of operation of the instrument can be obtained from the manufacturer.

3.5.2 Wilhelmy Plate Method

As opposed to the Maximum Bubble Pressure method, which measures a dynamic surface tension on a freshly made interface, the Wilhelmy Plate method (Adamson, 1990, Giannotta et al., 1993) measures an equilibrium surface tension on an "old" interface. The actual measurements were made on a DCA-322 system (CAHN Instruments Inc., Cerritos, CA) following the manufacturer's procedures. A platinum plate was used for the measurements and care was taken to make sure the plate was very clean. This technique measures the surface tension at equilibrium, or steady-state, which was typically 15 to 20 minutes after the solution interface was formed.

3.5.3 Visualization Of Bubbles

These experiments involved observing the adhesion of cells on the bubble surface through a microscopic lens. The experimental apparatus is very similar to those used by Garcia-Briones et al. (1992) and is shown in Figure 3.1.
Figure 3.1  Experimental apparatus for visualization of cell adhesion to the bubble surface.
The image recording equipment consisted of: a CCD video camera (Pulnix, model no. TM-640), an Infinity CFM lens, and a super VHS video recorder. A suitable light source was used such that the illumination allowed the cells to appear as white spots. The age of the bubbles ranged between 15 to 30 seconds on the solution surface. The volume of the cell suspension was 2 mL and air bubbles were introduced through the bottom of the column with a hypodermic needle. The diameter of the bubbles ranged between 3 to 3.5 mm, while the diameter of the cells were approximately 15 μm.

3.5.4 Bubble Rupture Experiments

With this experiment, the number of cells killed per bubble rupture was estimated. The experimental setup is similar to that used by Trinh et al. (1994). The method consisted of rupturing 400 bubbles in a 1 mL cell suspension using electrical sparks generated from an automobile coil and determining the change in total and viable cell counts. Using this apparatus, the relative protection provided by the different medium additives with respect to bubble rupture was determined.

3.5.5 Measurement Of Cell Number And Viability

The total cell concentration and the viable cell concentration were determined separately. It was observed that cells stained with Trypan blue can lyse soon after staining; consequently, total cell counts can not be based on the total number of stained and unstained cells and viability determinations should only be based on the number of unstained cells. For this reason, two independent cell counts are needed: one for the total number of cells present (without trypan blue), and one for the number of viable cells. The viable cell count was obtained by adding an equivalent amount of 0.1% w/v Trypan Blue solution in Hank’s balanced salt solution to the cell suspension. All cell rupture data was subjected to a Student’s t-test (99% confidence level).
3.6 Results

3.6.1 Measurement Of Interfacial Tensions

Table 3.3 presents data obtained by Maximum Bubble Pressure method and Wilhelmy Plate method for the TNMFH medium with and without additives. The values presented for the Wilhelmy Plate method are based on an average of four readings while the values for the Maximum Bubble Pressure method are based on an average of 500 readings of the same sample.

Table 3.4 presents data comparing the interfacial tensions of SFM-L and HBSS, with and without protective additives, obtained by Maximum Bubble Pressure method, as this method gives the dynamic value, which we believe to be more closely representative of the situation prevailing in an actual bioreactor.
Table 3.3
Interfacial Tensions Of Insect Cell Culture Media (TNMFH)
With And Without Protective Additives

<table>
<thead>
<tr>
<th>Additives</th>
<th>Interfacial tension (ergs/cm²) @ 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum Bubble Pressure method</td>
</tr>
<tr>
<td>No Additive</td>
<td>69.4±0.4</td>
</tr>
<tr>
<td>TNMFH with SF9 cells</td>
<td>69.8±0.2</td>
</tr>
<tr>
<td>Pluronic F68 @ 0.1% w/v</td>
<td>56.1±0.2</td>
</tr>
<tr>
<td>PVA @ 0.1% w/v</td>
<td>71.5±0.2</td>
</tr>
<tr>
<td>PEG (MW = 400) @ 0.1% w/v</td>
<td>70.7±0.2</td>
</tr>
<tr>
<td>PEG (MW = 1000) @ 0.1% w/v</td>
<td>67.6±0.1</td>
</tr>
<tr>
<td>PEG (MW = 4000) @ 0.1% w/v</td>
<td>65.7±0.2</td>
</tr>
<tr>
<td>Methocel E4M @ 0.3% w/v</td>
<td>-</td>
</tr>
<tr>
<td>Methocel E50 @ 0.3% w/v</td>
<td>55.5±0.6</td>
</tr>
<tr>
<td>Dextran @ 0.5% w/v</td>
<td>72.2±0.2</td>
</tr>
<tr>
<td>Dextran @ 5.0% w/v</td>
<td>71.9±0.2</td>
</tr>
</tbody>
</table>

Note: Methocel E4M has been reported to be surface-active (Sarkar, 1984). Data obtained for Methocel E4M has been influenced by the viscosity of the solution.
Table 3.4
Interfacial Tensions Of SFML And HBSS With And Without Protective Additives Measured By Maximum Bubble Pressure Method

<table>
<thead>
<tr>
<th>Additive</th>
<th>Interfacial tension (ergs/cm(^2)) @ 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SFM-L</td>
</tr>
<tr>
<td>No Additive</td>
<td></td>
</tr>
<tr>
<td>Pluronic F68 @ 0.1% w/v</td>
<td>57.2±0.2</td>
</tr>
<tr>
<td>PVA @ 0.1% w/v</td>
<td>63.4±0.2</td>
</tr>
<tr>
<td>PEG (MW = 400) @ 0.1% w/v</td>
<td>63.4±0.4</td>
</tr>
<tr>
<td>PEG (MW = 1000) @ 0.1% w/v</td>
<td>62.8±0.2</td>
</tr>
<tr>
<td>PEG (MW = 4000) @ 0.1% w/v</td>
<td>61.9±0.2</td>
</tr>
<tr>
<td>Methocel E4M @ 0.3% w/v</td>
<td>-</td>
</tr>
<tr>
<td>Methocel E50 @ 0.3% w/v</td>
<td>53.8±0.4</td>
</tr>
<tr>
<td>Dextran @ 0.5% w/v</td>
<td>63.1±0.2</td>
</tr>
<tr>
<td>Dextran @ 5.0% w/</td>
<td>64.4±0.4</td>
</tr>
</tbody>
</table>

Note: Methocel E4M has been reported to be surface-active (Sarkar, 1984). Data obtained for Methocel E4M has been influenced by the viscosity of the solution.
3.6.2 Visualization Of Bubbles At The Air-Medium Interface

As has been reported earlier, large numbers of cells suspended in TNMFH medium, without additives, adhere to the bubble film (Chalmers et al., 1991; Garcia-Briones et al., 1992). Cells suspended in SFM-L and HBSS, without additives, also adhered to the bubble film. Adhesion of cells to the bubble film in TNMFH medium without any additives is shown in Figure 2. At the other extreme, the presence of Pluronic F68 (0.1% w/v) and Methocel E50 (0.3% w/v) completely prevented the adhesion of cells to the bubble film, as presented in Figure 3a and 3b. The presence of other additives (PVA @ 0.1% w/v, PEG 4000 @ 0.1% w/v, and Dextran @ 5.0% w/v) in TNMFH produced results that were, qualitatively speaking, in between the two extremes discussed above (Figure 4a, 4b, and 4c). The observation with Methocel E4M lies in between that of Methocel E50 and PEG 4000. Though the data was obtained with TNMFH medium only, it is representative of the extent of cell-gas adhesion that can be observed with the other media (unpublished observations).
Figure 3.2 Photograph showing cells (SF9) attached to the bubble surface in TNMFH. An arrow indicates a particular cell.
Figure 3.3 Photographs showing cell adhesion to the bubble surface: a - SF9 cells in TNMFH plus 0.1% w/v Pluronic F68; b - SF9 cells in TNMFH plus 0.3% w/v Methocel E50.
Figure 3.4  Photographs showing cell adhesion to the bubble surface: a - SF9 cells in TNMFH plus 0.1% PVA; b - SF9 cells in TNMFH plus 0.1% PEG 4000.
Figure 3.4c  Photographs showing cell adhesion to the bubble surface: SF9 cells in TNMFH plus 5.0% Dextran.
3.6.3 Bubble Rupture Experiments

The results obtained from these experiments are shown in Table 3.5. The least amount of cell damage was observed with Pluronic F68, Methocel E50, and Methocel E4M in the medium. The other additives (PEG 4000, PVA, and Dextran) showed a low amount of protection. However, care should be taken in interpreting these results as is discussed below.
Table 3.5
Protection Efficiency Of Different Additives From Bubble Rupture Experiments

<table>
<thead>
<tr>
<th>Protective Agents</th>
<th>Cells Killed / Bubble</th>
<th>Degree Of Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>None</td>
<td>1600</td>
<td>2100</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>840</td>
<td>1100</td>
</tr>
<tr>
<td>Dextran</td>
<td>400</td>
<td>1300</td>
</tr>
<tr>
<td>PVA</td>
<td>390</td>
<td>730</td>
</tr>
<tr>
<td>Methocel E4M</td>
<td>70</td>
<td>50</td>
</tr>
<tr>
<td>Methocel E50</td>
<td>11</td>
<td>50</td>
</tr>
<tr>
<td>Pluronic F68</td>
<td>0</td>
<td>25</td>
</tr>
</tbody>
</table>

(Note: Methocel E4M has been reported to be surface-active (Sarkar, 1984). Data obtained for Methocel E4M has been influenced by the viscosity of the solution. Student's t-test (95% confidence level) was used to categorize the degree of protection offered by the different additives.)
3.7 Discussion

The results in Table 3.5 clearly indicate that Pluronic F68, Methocel E50, and Methocel E4M protect cells from single bubble rupture. The other additives, as shown in Table 3.5, also appear to protect cells, although, not as well as the above three. However, care should be taken in interpreting these results due to the large variability from one experiment to another, as shown from the results obtained with the two sets of experiments presented in Table 3.5. This variability was most clearly indicated in the work by Trinh et al. (1994) in which it was observed that on average 1150 cells were killed per bubble rupture in medium without any protective additives. This average was obtained from 8 separate experiments, with a standard deviation of 802 over a range of 335 to 2650 cells killed per bubble rupture. In the current studies, only two experiments were conducted for each additive. A Student's t-test (95% confidence level) showed that there is statistically significant difference with each of the additives, with respect to protection from bubble rupture, when compared to the control without any additives. However, the relative protection of PEG 4000, Dextran, and PVA is low when compared to Methocel E4M, Methocel E50, and Pluronic F68. Furthermore, there is no statistically significant difference between the protective attributes of PEG 4000, Dextran, and PVA.

Just as the method of determining the number of cells killed per single bubble rupture can only identify dramatic differences between additives, the visual observation of the number of cells on the bubble film can only be used to observe large differences. Again, the addition of Pluronic F68, Methocel E50, and Methocel E4M produced a statistically significant decrease in the number of cells on the bubble film.

In addition to cells remaining in the bubble film, cells have been observed to be attached to other gas-liquid interfaces such as the interface at the top of a bioreactor or the bottom of a bubble cavity (Bavarian et al., 1991; Chalmers et al., 1991). Trinh et al. (1994) reported that only about one third of the cells killed per bubble rupture were from
the bubble film. Since the fluid that experiences the highest hydrodynamic force originates in either the bubble film or the thin layer surrounding the bubble cavity, Trinh et al. (1994) concluded that if cells are excluded from both the bubble film and either the bubble cavity interface or the thin layer surrounding this cavity, no cell damage will occur. Cells on the solution surface away from the bubble cavity surface are not subjected to high hydrodynamic forces. When Pluronic F68, Methocel E50 and E4M were present, very little or no cell damage was observed with bubble rupture. From the above argument, and the observation that few if any cells were attached to the bubble film, it can be concluded that few if any cells were attached to the bubble cavity.

Based upon the photographic work of Bavarian et al (1991) and Chalmers et al. (1991) and the above results and discussion, the adhesion of cells to the air-liquid interface is non-specific in nature. This non-specific nature implies that cells do not differentiate between the different types of air-medium interfaces (such as, bubble lamella, bubble cavity, solution surface).

The three additives which provide the greatest protection also resulted in the greatest decrease in the dynamic $\gamma_{iv}$ (Table 3.3 and 3.4). The value of $\gamma_{iv}$ for Methocel E4M was not given because the instruments used in our laboratory were not capable of measuring $\gamma_{iv}$ in high viscosity solutions (Giannotta et al., 1993). The presence of Methocel E4M increases the viscosity of the solution from $1.2 \times 10^3$ N-s/m$^2$ to about $30 \times 10^3$ N-s/m$^2$ (Goldblum et al. (1990). However, it has been reported that Methocel E4M is surface-active in nature (Sarkar, 1984). This surface-active characteristic is also demonstrated by the fact that the only structural difference between E4M and E50, which did record a lowering of $\gamma_{iv}$, is the higher molecular weight of E4M (increased chain length).

An important observation, with respect to $\gamma_{iv}$, is the difference between the dynamic surface tension, as measured by the Maximum Bubble Pressure method, and the
equilibrium surface tension, as measured by the Wilhelmy plate method. These differences were expected. Several studies have shown that the diffusion, absorption, and denaturation of proteins at interfaces is a non-instantaneous process. At concentration much lower than in typical cell culture medium, BSA can take up to 30 minutes before it completely covers the interface (Narsimhan et al., 1992). The central question is: What is the characteristic time scale in a bioreactor with respect to bubble lifetime and cell-bubble interactions?

While it is difficult to determine a "typical" bubble diameter in a bioreactor, bubble sizes from 1 mm to 1 cm have been reported. These sizes correspond to bubble rise velocities of 14 cm/sec to 24 cm/sec (Brodkey, 1967). A typical height of a 2 liter bioreactor is 15 cm while that of a 20 L is 30 cm. This corresponds to bubble rise times of approximately 0.5 seconds to 1 second (if there is no bubble deflection in the impeller region). The dynamic $\gamma_n$ data reported using the Maximum Bubble Pressure method corresponds to characteristic time scales on the order of 1 second. The Wilhelmy plate method, however, determined $\gamma_n$ values on the order of 15 to 30 minutes. From this order of magnitude argument, it is apparent that the dynamic $\gamma_n$ is more relevant to the situation in a bioreactor than the equilibrium value obtained from the plate method. These observations also point to the complexity involved when dealing with interfacial phenomena, especially in a bioreactor with a large number of compounds present in the culture medium.

All of the results and discussion up to this point indicate that the additives which reduce the surface tension the most and the fastest (> 10 ergs/cm², less than 1 sec), as measured by the Maximum Bubble Pressure method, reduce or prevent cell adhesion to the gas-liquid interface and provide the greatest protection. The reduction in $\gamma_n$ can be related to the decrease in cell-gas adhesion by a thermodynamic equation, relating surface-energy to different interfacial tensions:
\[ \Delta F^{\text{adh}} = \gamma_{cv} - \gamma_{cl} - \gamma_{lv} \] (3.2).

This equation is obtained from an energy balance, and different forms of this equation, including equation (1), is already available in literature. While equation (1) is applicable to cell adhesion to a solid surface, the general relationship is applicable to any three phases, and in this case, the phases involved are: cell(c), liquid medium (l), and vapor (v) phase. As can be observed, a significant lowering in \( \gamma_{lv} \) could change the value of \( \Delta F^{\text{adh}} \) from a negative, thermodynamically favorable condition to a positive, thermodynamically unfavorable condition. It is reasonable to assume (although it has not been proven) that the interfacial tension between the cell and the vapor phase (\( \gamma_{cv} \)) for a particular cell-line will remain constant even if \( \gamma_{lv} \) is lowered and preliminary calculations have shown that cell-liquid interfacial tension (\( \gamma_{cl} \)) is low in magnitude (between 0 to 1 dyne/cm). Therefore, the value of \( \Delta F^{\text{adh}} \) will be most affected by a large change in \( \gamma_{lv} \).

It is important to emphasize that the value of \( \Delta F^{\text{adh}} \) is dependent on three interfacial tensions: \( \gamma_{lv} \), \( \gamma_{cv} \), and \( \gamma_{cl} \). In this paper, we have focused only on the study of \( \gamma_{lv} \) but that does not indicate that \( \gamma_{lv} \) is the only determining factor. The high protective action of Pluronic F68, Methocel E50, and Methocel E4M can be related to their surface-activity. The values of \( \gamma_{cv} \) and \( \gamma_{cl} \) and the effect, if any, that additives have on these values are being measured in current research in our laboratory.

In addition, we are not stating that the lowering of \( \gamma_{lv} \) is the only mechanism by which additives can protect cells. We are only stating that it is one of possibly several mechanisms. Interfacial phenomena, as stated above, is incredibly complex and difficult to characterize. This work is an attempt to develop a model that begins to explains its relationship to cell damage in bioreactors.
3.8 Conclusions

From the above observations, we propose that the change in interfacial properties through the addition of specific additives is one of the mechanisms by which suspended cells are protected from damage as a result of bubble rupture. In addition to lowering the interfacial tension of the medium, it is proposed that this lowering must be sufficiently rapid that it takes place before cells come in contact with the gas-liquid interface.

The non-specificity of the phenomenon of cell adhesion to the air-medium interface and the importance of the interfacial tensions has been further shown from the thermodynamic equation that relates the change in free energy for the process to the relevant three interfacial tensions, $\gamma_{lv}$, $\gamma_{cv}$, and $\gamma_{ci}$. In this work only $\gamma_{lv}$ was measured. In subsequent reports, the relationship of additives to other interfacial tensions will be presented.

The actual mechanism of cell damage in sparged bioreactors is highly complex and probably a function of several factors. This paper categorizes the additives used on the basis of a single bubble rupture in a small column. Another factor is the formation and continued presence of foam in a bioreactor, which Michaels and Papoutsakis (1991) have studied by determining the effectiveness of polyethylene glycol, polyvinyl alcohol and Pluronic F68 under such conditions. In contrast, no foam was present in any of the work presented in this paper.
CHAPTER IV
THERMODYNAMIC APPROACH TO EXPLAIN CELL ADHESION TO AIR-MEDIUM INTERFACES

The content of this chapter has been sent for publication in Biotechnology and Bioengineering.

4.1 Summary

Cell damage has been observed in suspension cell cultures with air sparging, especially in the absence of any protective additives. This damage is associated with cells adhering to bubbles and it has been shown that if this adhesion is prevented, cell damage is prevented. This paper presents a thermodynamic approach for predicting cell adhesion at the air-medium interface. With this relationship it can be shown that cell-gas adhesion can be prevented by lowering the surface tension of the liquid growth medium through the addition of surface-active protective additives. The thermodynamic relationship describes the change in free energy as a function of the interfacial tensions between the (i) gas and liquid phases, (ii) gas and cell phases, and (iii) liquid and cell phases. Experimental data, along with theoretical and empirical equations are used to quantify the changes in free energy that predict the feasibility of the process of cell-gas adhesion. The thermodynamic model is non-specific in nature and so results are equally valid for all types of cells.
4.2 Introduction

Air or oxygen can be supplied to a large-scale suspension cell culture through sparging. However, various studies have shown that sparging can result in cell damage in bioreactors, and the interaction between the cells and the bubbles formed during sparging is the responsible factor (Bavarian et al., 1991; Chalmers et al., 1991; Chattopadhyay et al. 1995a; Garcia-Briones et al., 1992; Handa-Corrigan et al., 1987, 1989; Jobses et al., 1990; Jordon et al., 1994; Kunas et al., 1990a; Murhammer et al., 1990a; Trinh et al., 1994). Microscopic and high-speed video methods (Bavarian et al., 1991; Chalmers et al., 1991) have indicated that cell damage is associated with cells adhering to or being very near to the bubble surface. Trinh et al. (1994) quantified the number of cells killed per bubble rupture at an air-medium interface and concluded that the cells killed were either attached to the bubble surface or were very near the surface. Trinh et al. (1994) also observed that the concentration of cells ejected as a bubble ruptured is higher than that in the bulk cell suspension; consequently, it was suggested that the concentration of cells in a very thin layer around the bubble is higher than that in the bulk suspension. This can best be explained if the cells are attached to the gas-liquid interface of the bubble. Then, once the bubble ruptures, the high hydrodynamic forces associated with the rupture kill these attached cells and eject some of these cells in the upward jet. Garcia-Briones et al. (1994) calculated the forces associated with the bubble rupture through computer simulations and demonstrated that these forces are several orders of magnitude greater than that required to damage cells. Therefore, in order to prevent cell damage, cell adhesion to the bubble surface, or to any air-medium interface, should be prevented.

In our earlier studies (Chattopadhyay et al., 1995a), we investigated the effect of protective additives on cell-gas adhesion and cell damage. It was shown through visualization studies that cells tend to attach to the air-medium interface, especially in the
absence of any additives. The number of cells damaged per bubble ruptured was maximum when there was a high degree of cell attachment to the bubble surface. However, in the presence of specific additives in the growth medium, such as Pluronic F68, Methocel E4M, and Methocel E50, the number of cells attached to the bubble surface was greatly reduced. The number of cells killed per bubble rupture were also greatly reduced in the presence of these additives. It was concluded that these protective additives prevent cell damage as a result of bubble rupture by preventing cell-gas adhesion. The presence of these additives lowered the surface tension of the medium (\(\gamma_{iv}\)) significantly indicating that these additives are surface-active in nature. Jordan et al. (1994) showed that the presence of Pluronic F68 in the medium prevented interaction between the cells and the bubbles, and they attributed this behavior to the surface-active nature of Pluronic F68.

Correlating the results obtained from the measurements of \(\gamma_{iv}\) of the medium in the presence of different additives and the degree of cell-gas adhesion (Chattopadhyay et al., 1995a), it was concluded that the additives which could rapidly lower the \(\gamma_{iv}\) (within a period of 1 second), also were more effective in preventing adhesion of cells to the bubble surface. This showed the importance of dynamic \(\gamma_{iv}\) measurements. The time required in the dynamic measurements is of the same order-of-magnitude as that of the life of a bubble in a typical bioreactor, and so the dynamic \(\gamma_{iv}\) is more relevant than the equilibrium value. Finally, it was observed that additives that do not rapidly lower \(\gamma_{iv}\), such as dextran, polyvinyl alcohol, polyethylene glycol, and serum, do not reduce either cell-gas adhesion or cell damage as a result of bubble rupture.

Finally, cell-gas adhesion was proposed to be dependent on non-specific factors (Chattopadhyay et al., 1995a), and this implies that a thermodynamic relationship can be used to determine the probability of the occurrence of cell-gas adhesion. Whether or not cell-gas adhesion occurs will depend on the magnitude of the change in free energy for
the process of cell-gas adhesion ($\Delta F_{\text{adh}}$), and $\Delta F_{\text{adh}}$ is dependent on various interfacial tensions ($\gamma$) involved in the system. This also indicates that the probability of the occurrence of the cell-gas adhesion can be altered by changing the various interfacial tensions.

### 4.3 Thermodynamic Equation For Cell-Gas Adhesion

The thermodynamic feasibility of a particular process can be determined from the direction of the change in free energy ($\Delta F$) taking place during the process. The process is thermodynamically feasible when $\Delta F$ is negative in value, and the process will be unfavorable when $\Delta F$ is positive. Interfacial tension ($\gamma$) is dependent on $\Delta F$ (Hiemenz, 1986). Interfacial tension ($\gamma$) has been defined as the work per unit area required to produce a new interface (dyne/cm) (Hiemenz, 1986). Interfacial tension ($\gamma$) has also been described as the specific surface free energy (ergs/cm$^2$) (Hiemenz, 1986). The change in free energy ($dF$), at constant temperature ($T$) and pressure ($P$), to create a new interface is equal to the non-pressure-volume work needed to create this interface (Hiemenz, 1986), and can be expressed as:

$$dF_{T,P} = \gamma.dA \quad (4.1)$$

where $dA$ is the increase in surface area.

Consider the case of a cell suspended in a bioreactor that is sparged with air as shown in Figure 4.1.
Figure 4.1  Schematic representation of the process of cell-gas adhesion in a bioreactor.
Three different phases coexist in the bioreactor: the cells (c), the vapor phase (v), and the liquid medium (l). There are, correspondingly, three different interfacial tensions: (i) interfacial tension between cell and vapor ($\gamma_{cv}$), (ii) interfacial tension between cell and liquid medium ($\gamma_{cl}$), and (iii) interfacial tension between liquid and vapor ($\gamma_{lv}$). Before a cell adheres to the gas-liquid interface, the cell and the bubble exist independently in the liquid medium, and the existing interfacial tensions are $\gamma_{cl}$ and $\gamma_{lv}$. After the cell attaches to the bubble surface, a new interface is formed between the cell and the bubble, and $\gamma_{cv}$ replaces $\gamma_{cl}$ and $\gamma_{lv}$ at that location. Therefore the change in specific free energy for the process of adhesion of cells to the bubble surface (vapor phase), with the liquid medium forming the surrounding phase is:

$$\Delta F^{adh} = \gamma_{final} - \gamma_{initial} = \gamma_{cv} - (\gamma_{lv} + \gamma_{cl})$$

(4.2)

Various forms of equation (4.2) have been discussed in the literature. For example, a form that relates to the adhesion of bacterial cells to various solid polymeric surfaces were studied by Absolom et al. (1983). In that system, the three interacting phases are: the solid polymeric surface (s), the bacterial cells on the solid surface (b), and the surrounding liquid (l). The $\Delta F^{adh}$ for the system is:

$$\Delta F^{adh} = \gamma_{bs} - \gamma_{bl} - \gamma_{sl}$$

(4.3)

Absolom et al. (1983) performed numerous experiments with different cells, liquids, and various solid surfaces to verify this relationship.

The thermodynamic relationship indicates the importance of the interfacial tensions in dictating the probability of the occurrence of cell-gas adhesion. In our earlier work (Chattopadhyay et al., 1995a), we have studied the effect of reduction in $\gamma_{lv}$ on cell-
gas adhesion. In this paper, we will be presenting data obtained for $\gamma_{cl}$ and $\gamma_{cv}$. Though these two interfacial tensions can not be measured directly, they can be determined by using semi-empirical equations.

4.4 Calculation Of Interfacial Tensions

In the early 19th century, Thomas Young proposed an equation that relates the different interfacial tensions in a three-phase solid-liquid-vapor system ($\gamma_{lv}$, $\gamma_{sv}$, and $\gamma_{sl}$) with the contact angle, $\theta$, the liquid makes on the solid surface:

$$\gamma_{lv} \cos \theta = \gamma_{sv} - \gamma_{sl}$$

(4.4)

Young's equation is strictly valid when the three phases (solid, liquid, and vapor) are in thermodynamic equilibrium with each other. This implies that Young's equation only applies at the line of contact where all three phases intersect. Elimination of the third phase can occur when the cells are prevented from attaching to the bubble surface. Another inherent assumption in Young's equation is that the liquid is in contact with a perfectly smooth solid surface. The consideration of an ideal surface neglects deviations that might occur due to roughness or chemical heterogeneity of the solid surface. However, in a biological system, especially where the liquid is in contact with the cells, the interface between the liquid and the cells (solid) is rough and chemically heterogeneous. Finally, it is assumed that the vapor in contact with the solid is saturated with the liquid; such ideal conditions may not exist, and to deal with these non-ideal conditions, additional terms can be introduced into Young's equation (Hiemenz, 1986), though these terms are generally difficult to evaluate. Due to such difficulties, for the present study, the bioreactor has been considered as an ideal system where the different phases obey Young's equation.
Of the four variables indicated in equation (4.4), only $\theta$ and $\gamma_v$ can currently be determined experimentally. Though the values of $\gamma_{cv}$ and $\gamma_d$ can not be determined directly, they can be calculated through the use of different available semi-empirical equations (Gerson, 1982; Neumann et al., 1974; Owen et al., 1969). The procedure to be followed for determining the values of $\gamma_{cv}$ and $\gamma_d$ is shown in Figure 4.2.
Young's Equation:
\[ \gamma_{cv} - \gamma_{cl} = \gamma_{lv} \cos \theta \]

Semi-empirical Equations
\[ \gamma_{cv} = f(\gamma_{lv}, \theta) \]

Experimental data of \( \gamma_{lv} \) & \( \cos \theta \)

\( \gamma_{cv} \) & \( \gamma_{cl} \)

\[ \Delta F^{ad} = \gamma_{cv} - \gamma_{lv} - \gamma_{cl} \]

Figure 4.2 Schematic representation for the procedure followed for the calculation of \( \gamma_{cv} \) and \( \gamma_{cl} \).
Experimentally determined values of $\gamma_v$ and $\theta$ are required in the calculation of $\gamma_{sv}$ and $\gamma_{sl}$. Finally, $\Delta F^{\text{adh}}$ can be calculated (equation 4.2) from the three different interfacial tensions.

### 4.4.1 Semi-Empirical Equations Of State

Three sets of semi-empirical equations (Gerson, 1982; Neumann et al., 1974; Owen et al., 1969) have been reviewed. For all of these equations, the cells have been considered as the solid phase. One of the first set of equations for determination of $\gamma_{sl}$ and $\gamma_{sv}$ was proposed by Owen and Wendt (1969). According to Owen and Wendt (1969), the value of $\gamma_{sv}$ can be calculated from $\theta$ and $\gamma_v$ as:

$$\gamma_v (1 + \cos \theta) = 2(\sqrt{\gamma_{sv}^d \gamma_{sv}^d} + \sqrt{\gamma_{sv}^h \gamma_{sv}^h})$$

(4.5)

In equation (4.5), $\theta$ and $\gamma_v$ for two different liquids on the same solid surface have to be determined experimentally to obtain the values of $\gamma_{sv}^d$ and $\gamma_{sv}^h$. Summation of $\gamma_{sv}^d$ and $\gamma_{sv}^h$ will provide $\gamma_{sv}$. The value of $\gamma_{sl}$ can be determined by using Young’s equation (equation 4.4) with equation (4.5) as

$$\gamma_{sl} = \gamma_{sv} + \gamma_v - 2\sqrt{\gamma_{sv}^d \gamma_{sv}^d} - 2\sqrt{\gamma_{sv}^h \gamma_{sv}^h}$$

(4.6)

Neumann et al. (1974) proposed another set of equations to provide a relationship between the three interfacial tensions ($\gamma_{sv}$, $\gamma_{sl}$, and $\gamma_v$) and the contact angle ($\theta$) in a solid-liquid-vapor system. The advantage of this method is that only one liquid can be used to obtain the necessary data, unlike the method proposed by Owen and Wendt (1969). The value of $\gamma_{sv}$ can be determined from the experimentally determined values of $\gamma_v$ and $\theta$ by using the following equation:
\[ \cos \theta = \frac{(0.015 \gamma_{sv} - 2.0) \sqrt{\gamma_{sv} \gamma_{lv}} + \gamma_{lv}}{\gamma_{lv} [0.015 \sqrt{\gamma_{sv} \gamma_{lv}} - 1]} \]  
(4.7)

With the calculated value of \( \gamma_{sv} \), \( \gamma_{si} \) can be determined by substituting Young's equation (equation 4.4) in equation (4.7):

\[ \gamma_{si} = \frac{\left( \gamma_{sv} \right)^{\frac{1}{2}} - \left( \gamma_{lv} \right)^{\frac{1}{2}}}{1 - 0.015 \left( \gamma_{sv} \gamma_{lv} \right)^{\frac{1}{2}}} \]  
(4.8)

In 1982, Gerson developed a set of equations, which is actually an improvement over those proposed by Neumann et al. (1974). In this case there are two equations: the first one for the determination of \( \gamma_{sv} \) from experimentally determined values of \( \gamma_{lv} \) and \( \theta \) as:

\[ \frac{\gamma_{lv} (\cos \theta + 1)}{2 \sqrt{\gamma_{lv} \gamma_{sv}}} - \exp[(\gamma_{sv} - \gamma_{lv} \cos \theta)(a \gamma_{sv} + b)] = 0 \]  
(4.9),

and the second equation, obtained by substituting Young's equation (equation 4) in equation (4.9) allows calculation of \( \gamma_{si} \) from \( \gamma_{lv} \) and \( \gamma_{sv} \):

\[ \frac{\gamma_{lv} + \gamma_{sv} - \gamma_{si}}{2 \sqrt{\gamma_{lv} \gamma_{sv}}} - \exp(\gamma_{si}(a \gamma_{sv} + b)) = 0 \]  
(4.10).

The above two equations can be solved numerically with the best-fit values of the parameters: \( a = 6.5 \pm 1.0 \times 10^{-3} \) and \( b = -0.010 \pm 0.001 \), which were provided by the author (Gerson, 1982). These parameters were obtained from testing a variety of systems (solid, liquid, and vapor components) from a number of published literature. Derivations
of these semi-empirical equations are shown in Section 4.10.

In this study, the $\Delta F^{adh}$ for cell-gas adhesion of several cell lines has been determined, and the equations proposed by Neumann et al. (1974) and Gerson (1982) has been used to calculate $\gamma_{cv}$ and $\gamma_{cl}$.

4.5 Materials Used

Four different cell lines were used: Spodoptera frugiperda (SF9) (Chattopadhyay et al., 1995a) cells, Chinese Hamster Ovary (CHOK1, ATCC-CCL 61) cells, hybridoma cells (HT24, obtained from Dr. Richard F. Mortensen, Department of Microbiology, The Ohio State University, Columbus, OH), and normal African green monkey cells (N-VERO cells - ATCC CCL-81). The SF9 cells were subcultured in five different media/additive combinations: (i) serum-containing medium (TNMFH, Sigma Chemical Co., MO) with 10% v/v fetal bovine serum (FBS) without any additives, (ii) TNMFH with 0.1% w/v Pluronic F68 (BASF Corporation, NJ), (iii) TNMFH with 0.3% w/v Methocel E50 (Dow Chemical Co., Midland, MI), (iv) serum-free media supplemented with lipids (SFML) (Hink, 1991) (obtained from Dr. Fred Hink, Department of Entomology, The Ohio State University), and (v) SFML with 0.1% w/v Pluronic F68. Ham F12 (Sigma Chemical Co., MO) medium containing FBS (10% v/v) was used for the CHOK1 cells, and RPMI (Sigma Chemical Co., MO) medium containing FBS (10% v/v) was used for the hybridomas. The N-VERO cells were subcultured in Medium 199 (M199) (Sigma Chemical Co., MO) with FBS (10% v/v). The contact angles on all the cell monolayers were measured using Hanks' balanced salt solution (Chattopadhyay et al., 1995a) (HBSS) and/or the respective culture medium. HBSS and/or the respective culture medium with 0.1% w/v Pluronic F68 was also used for measuring contact angles on CHOK1 cells, N-VERO cells, and SF9 cells.
4.6 Experimental Techniques

As reported above, two types of experimental data are needed to calculate $\gamma_{ov}$ and $\gamma_{ol}$, and to determine $\Delta F^{adh}$: the contact angle ($\theta$) that a drop of liquid makes on a monolayer of cells and the interfacial tension of that liquid, $\gamma_{iv}$. Two different techniques were used (Maximum Bubble Pressure method and Wilhelmy plate method) to obtain values of $\gamma_{iv}$ and those values were reported in our earlier work (Chattopadhyay et al., 1995a). For the present study, the $\gamma_{iv}$ of RPMI medium was determined by the Maximum Bubble Pressure method (Chattopadhyay et al., 1995a) only, whereas the $\gamma_{iv}$ values of Ham F12 and M199 media were determined by both the Maximum Bubble Pressure method and the Wilhelmy plate methods. A modified version of the Axisymmetric Drop Shape Analysis (ADSA) (Duncan-Hewitt et al., 1989) method was used to determine the contact angle that a liquid drop makes on a monolayer of cells and the procedure followed is described below. For two of the cell lines (CHOK1 and N-VERO), the contact angles were also determined by using the Wilhelmy plate method, as this method was successful only in the case of anchorage-dependent cells.

4.6.1 Axisymmetric Drop Shape Analysis (ADSA)

The ADSA method, initiated by Rotenberg et al. (1982), is based on the shape of static drops on a substrate (Rotenberg et al., 1982, Duncan-Hewitt et al., 1989). The main advantage of this method is that a small quantity of liquid can be used to obtain the data with a high degree of accuracy. A modified version of this method (ADSA-CD) was used by Duncan-Hewitt et al. (1989) to measure the contact angle of liquid on monolayers of bacterial cells. The ASDA-CD method is specifically suitable for systems with low contact angles, as in the case of biological systems. The computer program ADSA-CD (Department of Mechanical Engineering, University of Toronto, Canada) was used to calculate the values of $\theta$. 
The ADSA-CD technique uses measurements of the average contact diameter, as viewed from above, of a sessile drop on a surface to determine the contact angle that the drop makes with the surface. These measurements are made with small volumes of liquid under conditions such that the gravitational and the surface tensional effects are comparable. Other data needed for this technique are the volume of the drop, the surface tension of the liquid ($\gamma_w$) used for measuring the contact angle, the difference in the densities of the two contacting phases (in this cases they are the liquid and the vapor phase), and the gravitational constant (g).

The procedure followed in the present study to obtain the contact diameter of a drop on a monolayer of cells (Absolom et al., 1983, 1986; Duncan-Hewitt et al., 1989) is outlined below. The cells were grown in a special cell culture chamber (Lab-Tek Chamber Slide - Tissue Culture Chambers, Fisher Scientific) which contained a microscope type slide. After the slide became confluent with the cells, the medium was drained off and the cells were washed with HBSS. Sufficient time (6 to 10 min) was given to evaporate the extraneous liquid before using the cells for experiments. Precautions were taken such that the cells do not become excessively dry. A drop of known volume of the culture medium of the particular cell-line or HBSS (3 to 5μL) was then placed on the cell surface and the image of this drop was recorded on a VCR using a CCD video camera (Pulnix - model no. TM-640). Figure 4.3 shows the experimental apparatus used.
Figure 4.3 Experimental apparatus used for the determination of contact angles by the ADSA-CD technique.
The major challenge in using this method is its requirement of accurate coordinate points of the drop perimeter. To acquire the coordinates of the drop, digital image processing was employed. A frame grabber (Dipix P360 frame grabbing board in a PC) was used to convert the analog video signal to a digital signal. The pixel coordinates of the drop were then picked manually using a specifically written computer program. These values, calibrated to real world coordinates, were used to determine the contact diameter of the drop. A precisely manufactured grid (Lovins Micro-slide Field Finder, Teledyne Gurley, NY) was used for the calibration.

In a separate experiment, CHOK1 cells were cultivated in Ham F12 medium which was then replaced with HBSS containing 0.1% w/v Pluronic F68 ($\gamma_v$ (Chattopadhyay et al., 1995a) = 58.0±0.1 ergs/cm$^2$). After about 90 minutes the contact angle on this monolayer of cells was measured with HBSS containing 0.1% w/v Pluronic F68. Similar experiment was also conducted with the N-VERO and the SF9 cells. Culture media containing 0.1% Pluronic F68 were also used to determine the contact angles on these cells. Spreading of the liquid (culture medium / HBSS + 0.1% w/v Pluronic F68) on the cell monolayer was observed (unable to form a sessile drop) in all the cases.

4.6.2 Wilhelmy Plate Method

The Wilhelmy Plate method for obtaining $\gamma_v$ has already been described in our previous paper (Chattopadhyay et al., 1995a). The same method has been used for this study in obtaining values of contact angles. Cells were allowed to grow on both sides of a microscope cover slide, and once both sides become confluent with cells, the slide was used for determining $\theta$. In order to ensure that the cells do not come off the surface of the cover slide during the measurements, only the anchorage dependent cell lines, CHOK1 and N-VERO, were used. The liquids used in these measurements were Ham F12 and
M199 media for CHOK1 and N-VERO, respectively.

4.6.3 Justification For The Use Of HBSS

The main purpose of using HBSS in our experiments was to use a defined medium for the measurement of contact angles so that the surface properties of the cells will not be masked by other molecules, such as proteins and serum, that are usually present in a growth medium. Before the experiments, we have also washed the cells with HBSS to remove any adhering proteins or serum from the cell surface. Moreover, different cells use different culture medium, and use of HBSS for all the cells helped us in comparing the values of contact angles on a similar basis. The measurements of contact angles were conducted to determine $\gamma_{cv}$ of the cells, and as this is a property of the particular cell surface, it should not be influenced by the liquid used in measurements. Finally, experiments have also been conducted with the respective growth medium, and there exists no difference between the results obtained with HBSS and with growth medium.

4.7 Results

Table 4.1 presents the values of surface tension ($\gamma_{lv}$), and the method used to calculate the surface tension, for the various liquids used in this study. The actual procedure along with some of this data was reported in our previous publication (Chattopadhyay et al., 1995a) on this area of research.
Table 4.1

Surface Tension Values Of Medium

<table>
<thead>
<tr>
<th>Medium</th>
<th>$\gamma_v$ (ergs/cm$^2$)</th>
<th>Method of Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>72.8 ± 0.4</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td>TNMFH</td>
<td>69.4 ± 0.4</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td>TNMFH + 0.1% PF68</td>
<td>56.1 ± 0.2</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td>TNMFH + 0.3% ME50</td>
<td>55.5 ± 0.6</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td>SFML</td>
<td>64.0 ± 0.3</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td>SFML + 0.1% PF68</td>
<td>57.2 ± 0.2</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td>Ham F12</td>
<td>69.2 ± 0.2</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td></td>
<td>48.1 ± 0.6</td>
<td>Wilhelmy Plate</td>
</tr>
<tr>
<td>M199</td>
<td>68.9 ± 0.8</td>
<td>Maximum Bubble Pressure</td>
</tr>
<tr>
<td></td>
<td>50.1 ± 1.2</td>
<td>Wilhelmy Plate</td>
</tr>
<tr>
<td>RPMI</td>
<td>69.1 ± 0.6</td>
<td>Maximum Bubble Pressure</td>
</tr>
</tbody>
</table>

Note: HBSS = Hank's balanced salt solution, PF68 = Pluronic F68, ME50 = Methocel E50.
Table 4.2 presents the values of contact angles for different cell lines, grown in their respective medium, obtained by using the ADSA-CD technique or Wilhelmy Plate method with various test liquids. For all of the cells, except for hybridoma, the liquid used to determine the contact angle was the appropriate culture medium, and for comparison between cells, HBSS was used as the test liquid. In all the cases, there was no statistical difference between the contact angles obtained by using the culture medium versus HBSS. The following values were also used in the calculation of $\theta$ using the ADSA-CD program: $\Delta \rho$ (difference in density between the liquid and the vapor phase) for HBSS = 1.0072 g/mL, $\Delta \rho$ for TNMFH = 1.0135 g/mL, $\Delta \rho$ for Ham F12 = 1.0107 g/mL, $\Delta \rho$ for M199 = 1.0129 g/mL.
Table 4.2

Experimental Values Of Contact Angles Of Cell Lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Growth medium</th>
<th>Test liquid</th>
<th># of data points</th>
<th>θ, deg.</th>
<th>Range of θ, deg.</th>
<th>Avg. θ, deg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF9</td>
<td>TNMFH</td>
<td>HBSS</td>
<td>12</td>
<td>12±2(a)</td>
<td>10-15</td>
<td>13±2(b)</td>
</tr>
<tr>
<td></td>
<td>TNMFH</td>
<td>HBSS</td>
<td>9</td>
<td>15±2(a)</td>
<td>13-17</td>
<td></td>
</tr>
<tr>
<td>SFML</td>
<td>HBSS</td>
<td>47</td>
<td>12±5(a)</td>
<td>6-21</td>
<td>12±5</td>
<td></td>
</tr>
<tr>
<td>TNMFH+0.3%</td>
<td>HBSS</td>
<td>8</td>
<td>13±4(a)</td>
<td>9-17</td>
<td>13±4</td>
<td></td>
</tr>
<tr>
<td>ME50</td>
<td>HBSS</td>
<td>10</td>
<td>8±2(a)</td>
<td>6-11</td>
<td>8±2</td>
<td></td>
</tr>
<tr>
<td>TNMFH+0.1%</td>
<td>HBSS</td>
<td>27</td>
<td>7±2(a)</td>
<td>5-11</td>
<td>7±2</td>
<td></td>
</tr>
<tr>
<td>SFML+0.1%</td>
<td>HBSS</td>
<td>27</td>
<td>7±2(a)</td>
<td>5-11</td>
<td>7±2</td>
<td></td>
</tr>
<tr>
<td>SFML+0.1%</td>
<td>HBSS</td>
<td>27</td>
<td>7±2(a)</td>
<td>5-11</td>
<td>7±2</td>
<td></td>
</tr>
<tr>
<td>CHOK1</td>
<td>Ham F12</td>
<td>HBSS</td>
<td>39</td>
<td>13±6(a)</td>
<td>6-23</td>
<td>14±5(b)</td>
</tr>
<tr>
<td></td>
<td>Ham F12</td>
<td>HBSS</td>
<td>31</td>
<td>16±2(a)</td>
<td>13-19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ham F12</td>
<td>HBSS</td>
<td>6</td>
<td>34±4(1)</td>
<td>31-37</td>
<td>27±9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19±2(2)</td>
<td>17±21</td>
</tr>
<tr>
<td>N-VERO</td>
<td>M199</td>
<td>HBSS</td>
<td>14</td>
<td>29±3(a)</td>
<td>26-34</td>
<td>29±3(b)</td>
</tr>
<tr>
<td></td>
<td>M199</td>
<td>HBSS</td>
<td>20</td>
<td>29±4(a)</td>
<td>20-34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M199</td>
<td>HBSS</td>
<td>6</td>
<td>22±5(1)</td>
<td>17-27</td>
<td>18±6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14±6(2)</td>
<td>11-20</td>
</tr>
<tr>
<td>Hyb.</td>
<td>RPMI</td>
<td>HBSS</td>
<td>18</td>
<td>9±3(a)</td>
<td>5-12</td>
<td>9±3</td>
</tr>
</tbody>
</table>

Note: Hyb. = Hybridoma, PF68 = Pluronic F68, ME50 = Methocel E50, a = γ determined by ADSA-CD technique, 1 = advancing angle (determined by Wilhelmy plate method), 2 = receding angle (determined by Wilhelmy plate method), b = no statistically significant difference (Student's t-test) between the readings obtained with HBSS and the culture medium
When Pluronic F68 was present in the test liquid (either culture medium or HBSS), complete "wetting" was observed and it was impossible to calculate a finite contact angle. Consequently, in these cases, the contact angle can be assumed to be zero. To determine if the growth of cells in medium containing additives changed the contact angle, SF9 cells were grown in TNMFH medium containing Pluronic F68 and Methocel E50. The cells were also grown in SFML medium and SFML medium containing Pluronic F68. A small, but statistically significant difference was observed when the cells were grown in the presence of Pluronic F68. However, no difference was observed with the cells grown in the presence of Methocel E50.

Significant differences in the contact angles were observed when the data obtained using the Wilhelmy Plate method is compared to the data obtained with the ADSA-CD technique. In addition, differences were observed when an advancing versus receding angle was determined. However, the differences varied based on the cell lines. In the case of the CHOK1 cells, the Wilhelmy Plate method gave values which were higher than that obtained with the ADSA-CD technique, while in the case of the N-VERO cells, the Wilhelmy Plate method gave values which were lower than ADSA-CD method. For both cell lines, the receding angles were lower than the advancing angle.

With the experimentally determined values of \( \theta \) and \( \gamma_{lv} \), Young's equation, and the semi-empirical equations of state, values of \( \gamma_{cw} \) and \( \gamma_{dl} \) were calculated for the different cells and media combinations. Since it was not possible to determine the relative accuracy of each set of semi-empirical equations, the calculations were repeated with the equations proposed by both Neumann et al. (1974) and Gerson (1982). The equations proposed by Owen and Wendt (1969) were not used in the calculations since we do not have all the required data. Tables 4.3 and 4.4 present the data for the different cell lines tested in our laboratory, along with the experimentally determined contact angles, the interfacial tensions of the typical growth media with and without additives, the calculated
values of $\gamma_{ev}$ and $\gamma_{cd}$, and the corresponding $\Delta P_{adh}$ values.
### Table 4.3
Calculation Of $\gamma_{cv}$ and $\Delta F_{adh}$ Using Experimental Data Obtained With ADSA-CD Technique

<table>
<thead>
<tr>
<th>Cell Line/Growth Medium</th>
<th>Contact Angle, $\theta$ (degrees)</th>
<th>$\eta_v$ of growth medium</th>
<th>$\gamma_{cv}^*$</th>
<th>$\gamma_{cl}^*$</th>
<th>$\Delta F_{adh}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF9/TNMFH</td>
<td>13±2</td>
<td>69.4±0.4</td>
<td>67.1-68.2(a)</td>
<td>0.02-0.08(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>67.1-68.1(b)</td>
<td>0.02-0.08(b)</td>
<td></td>
</tr>
<tr>
<td>SF9/TNMFH + 0.1% PF68</td>
<td>8±2</td>
<td>56.1±0.2</td>
<td>68.4-69.0(a)</td>
<td>0.002-0.02(a)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>68.4-69.0(b)</td>
<td>0.002-0.02(b)</td>
<td></td>
</tr>
<tr>
<td>SF9/TNMFH + 0.3% ME50</td>
<td>13±4</td>
<td>55.5±0.6</td>
<td>66.5-68.6(a)</td>
<td>0.01-0.13(a)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66.5-68.6(b)</td>
<td>0.01-0.13(b)</td>
<td></td>
</tr>
<tr>
<td>SF9/SFML</td>
<td>12±5</td>
<td>64.0±0.3</td>
<td>61.3-63.5(a)</td>
<td>0.003-0.1(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61.3-63.5(b)</td>
<td>0.003-0.12(b)</td>
<td></td>
</tr>
<tr>
<td>SF9/SFML + 0.1% PF68</td>
<td>7±2</td>
<td>57.2±0.2</td>
<td>63.2-63.8(a)</td>
<td>0.001-0.01(a)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63.2-63.8(b)</td>
<td>0.001-0.01(b)</td>
<td></td>
</tr>
<tr>
<td>CHOK1/Ham F12</td>
<td>14±5</td>
<td>69.2±0.3</td>
<td>65.6-68.4(a)</td>
<td>0.01-0.2(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65.6-68.4(b)</td>
<td>0.01-0.21(b)</td>
<td></td>
</tr>
<tr>
<td>N-VERO/M199</td>
<td>29±3</td>
<td>68.9±0.8</td>
<td>59.7-62.6(a)</td>
<td>0.63-1.31(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>59.9-62.6(b)</td>
<td>0.68-1.45(b)</td>
<td></td>
</tr>
<tr>
<td>Hybridoma/RPMI</td>
<td>9±3</td>
<td>69.1±0.6</td>
<td>67.6-68.7(a)</td>
<td>0.002-0.03(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>67.6-68.7(b)</td>
<td>0.002-0.03(b)</td>
<td></td>
</tr>
</tbody>
</table>

Note: a = Determined by Neumann's equations; b = Determined by Gerson's equations, * = values of $\gamma_{cv}$ and $\gamma_{cl}$ were calculated based on values of $\gamma$ of the regular medium without any additive.
### Table 4.4

**Calculation Of $\gamma_{CV}$ and $\Delta \gamma_{adh}$ Using Experimental Data Obtained With Wilhelmy Plate Method**

<table>
<thead>
<tr>
<th>Cell Line/Growth Medium</th>
<th>Contact Angle, $\theta$ (degrees)</th>
<th>Test liquid</th>
<th>$\gamma_{IV}$ of growth medium</th>
<th>$\gamma_{CV}^*$</th>
<th>$\gamma_{CL}^*$</th>
<th>$\Delta \gamma_{adh}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOK1/Ham F12</td>
<td>34±4(adv.)</td>
<td>Ham F-12</td>
<td>69.2±0.3</td>
<td>56.9-61.0 (a)</td>
<td>1.1-2.4</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>57.2-61.1 (b)</td>
<td>1.2-2.7</td>
<td>(-)</td>
</tr>
<tr>
<td>CHOK1/Ham F12</td>
<td>19±2(rec.)</td>
<td></td>
<td>69.2±0.3</td>
<td>64.9-66.3 (a)</td>
<td>0.13-0.29</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>64.9-66.3 (b)</td>
<td>0.13-0.30</td>
<td>(-)</td>
</tr>
<tr>
<td>CHOK1/Ham F12</td>
<td>27±9(avg.)</td>
<td></td>
<td>69.2±0.3</td>
<td>58.0-66.0 (a)</td>
<td>0.16-2.0</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>58.2-66.0 (b)</td>
<td>0.17-2.2</td>
<td>(-)</td>
</tr>
<tr>
<td>N-VERO/M199</td>
<td>22±5(adv.)</td>
<td>M199</td>
<td>68.9±0.8</td>
<td>62.1-66.0 (a)</td>
<td>0.13-0.72</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>62.2-66.0 (b)</td>
<td>0.13-0.78</td>
<td>(-)</td>
</tr>
<tr>
<td>N-VERO/M199</td>
<td>14±6(rec.)</td>
<td></td>
<td>68.9±0.8</td>
<td>65.0-68.2 (a)</td>
<td>0.007-0.24</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65.0-68.2 (b)</td>
<td>0.007-0.25</td>
<td>(-)</td>
</tr>
<tr>
<td>N-VERO/M199</td>
<td>18±6(avg.)</td>
<td></td>
<td>68.9±0.8</td>
<td>63.4-67.4 (a)</td>
<td>0.03-0.47</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>63.4-67.4 (b)</td>
<td>0.03-0.50</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Note: $a = \text{Determined by Neumann's equations;}$ $b = \text{Determined by Gerson's equations;}$ $^* = \text{values of } \gamma_{CV}$ and $\gamma_{CL}$ were calculated based on values of $\gamma_{IV}$ of the regular medium without any additive.
In addition to the experimental data obtained in our laboratory, reported data (Absolom et al., 1983, 1986; Duncan-Hewitt et al., 1989) on θ of a known liquid on monolayers of different cells were also used to calculate γ_{cv}, γ_{cd}, and ΔF^{adh} and these results are presented in Table 4.5. In all the cases (Tables 4.3, 4.4 and 4.5) the γ_{cv}, and γ_{cd} were calculated by the equations proposed by Neumann et al. (1974) and Gerson (1982) equations, and ΔF^{adh} was calculated by using equation (4.2).
Table 4.5

Calculation Of $\gamma_{CV}$ and $\Delta F_{adh}$ Using Data Obtained From Literature

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Contact Angle, $\theta$</th>
<th>$\gamma_V$ (test liquid)</th>
<th>$\gamma_{CV}$</th>
<th>$\gamma_{cl}$</th>
<th>$\Delta F_{adh}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>guinea pig neutrophil</td>
<td>13.1</td>
<td>72.6</td>
<td>70.8(a)</td>
<td>0.06(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>70.8(b)</td>
<td>0.05(b)</td>
<td>(-)</td>
</tr>
<tr>
<td>guinea pig alveolar macrophage</td>
<td>21.4</td>
<td>72.6</td>
<td>67.95(a)</td>
<td>0.36(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>67.95(b)</td>
<td>0.36(b)</td>
<td>(-)</td>
</tr>
<tr>
<td>human neutrophil</td>
<td>18.4</td>
<td>72.6</td>
<td>69.1(a)</td>
<td>0.2(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>69.1(b)</td>
<td>0.2(b)</td>
<td>(-)</td>
</tr>
<tr>
<td>human lymphocytes</td>
<td>15.6</td>
<td>72.6</td>
<td>70.0(a)</td>
<td>0.11(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>70.0(b)</td>
<td>0.10(b)</td>
<td>(-)</td>
</tr>
<tr>
<td><em>E. coli</em> 055*</td>
<td>16.7±1.0</td>
<td>69.7±0.4</td>
<td>66.6-67.2(a)</td>
<td>0.10-0.16(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66.6-67.2(b)</td>
<td>0.10-0.16(b)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

(contd.)
Table 4.5 (contd.)

**Calculation Of $\gamma_{cv}$ and $\Delta \gamma_{padh}$ Using Data Obtained From Literature**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Contact Angle, $\theta$</th>
<th>$\eta_{lv}$ (test liquid)</th>
<th>$\gamma_{cv}$</th>
<th>$\gamma_{cl}$</th>
<th>$\Delta \gamma_{padh}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli 2627</em></td>
<td>21.2±0.7</td>
<td>67.9±0.3</td>
<td>63.3-63.85(a)</td>
<td>0.25-0.32(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63.3-63.9(b)</td>
<td>0.27-0.35(b)</td>
<td>(-)</td>
</tr>
<tr>
<td><em>S. aureus 049</em></td>
<td>18.5±1.2</td>
<td>69.1±0.6</td>
<td>65.3-66.1(a)</td>
<td>0.14-0.23(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65.3-66.1(b)</td>
<td>0.14-0.24(b)</td>
<td>(-)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>23.4±0.5</td>
<td>67.1±0.3</td>
<td>61.8-62.2(a)</td>
<td>0.37-0.43(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61.8-62.2(b)</td>
<td>0.40-0.47(b)</td>
<td>(-)</td>
</tr>
<tr>
<td>PK-15*</td>
<td>22.0±3.0</td>
<td>64.0±3.0</td>
<td>58.45-60.7(a)</td>
<td>0.16-0.45(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58.5-60.7(b)</td>
<td>0.18-0.51(b)</td>
<td>(-)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>26.1±1.2</td>
<td>66.3±0.6</td>
<td>59.6-60.6(a)</td>
<td>0.48-0.67(a)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>59.7-60.7(b)</td>
<td>0.54-0.75(b)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

Note: a = Determined by Neumann's equations; b = Determined by Gerson's equations.
4.8 Discussion

A fundamental assumption in Young's equation is that the three phases (vapor, liquid, and cell) are in contact and at equilibrium with each other. An implication of this assumption is that the semi-empirical equations used in this study to calculate $\gamma_{cv}$ and $\gamma_{cl}$ from $\theta$ and $\gamma_v$ will only give meaningful results if the three phases are in contact and at equilibrium. Consequently, meaningful data was obtained only when stable sessile drops were formed by the liquid on a monolayer of cells. Stable sessile drops formed only when surface-active protective additives (Pluronic F68 and Methocel E50) were not present in the medium, which means the liquid used had a high surface tension. When Pluronic F68, which reduces $\gamma_v$, was present in the test liquid (both in the respective culture medium as well as HBSS), complete wetting was observed which corresponds to a zero contact angle condition. For this reason, all contact angle experiments were conducted with HBSS or culture medium without any additives.

A zero contact angle condition indicates a tendency towards the elimination of the third phase (vapor phase), and under such a condition $\Delta F_{\text{adh}} \geq 0$. This results confirms that equation (4.2) is qualitatively correct in explaining the phenomenon of prevention of cell-gas adhesion in the presence of protective additives (Pluronic F68 and Methocel E50). The remainder of this paper discusses our attempt to quantify this relationship. It is very difficult to quantitatively measure interfacial properties in complex systems such as biological systems. Nevertheless, attempts have been made to measure these quantities with some degree of accuracy (Absolom et al., 1983, 1986; Duncan-Hewitt et al., 1989, Hato et al., 1985). In this work, several of these techniques, both theoretical and experimental were used for this purpose.

As mentioned earlier, the feasibility of the process of cell-gas adhesion can be determined from the magnitude of $\Delta F_{\text{adh}}$. In order to determine $\Delta F_{\text{adh}}$ from equation (4.2), values of all the interfacial tensions are required. The values of $\gamma_v$ were determined
experimentally, while the values of $\gamma_{cv}$ and $\gamma_{cl}$, were calculated from the experimental data. As shown in Tables 4.3 and 4.4, $\Delta F^{adh}$ is negative when protective additives, such as Pluronic F68 and Methocel E50, were absent. This indicates that cell adhesion to a gas-liquid interface will occur, and this is consistent with the experimental observations that insect cells (Chattopadhyay et al., 1995a), CHOK1 and hybridoma cells (personal observations) adhere to gas-liquid interfaces in the absence of any additives. However, in the presence of these additives, $\Delta F^{adh}$ becomes positive indicating that the process of cell-gas adhesion will not occur. This also corroborates with observations reported earlier (Chattopadhyay et al., 1995a).

The values of contact angles made by a biologically compatible liquids on cell surfaces, for 14 different cell lines, and 18 different medium-cell combinations, are low (Tables 4.2 and 4.5). It is also found that the values of $\gamma_{cv}$ and $\gamma_{cl}$ (Tables 4.3, 4.4 and 4.5), as determined from two sets of semi-empirical equations (Gerson, 1982; Neumann et al., 1974), are similar. The $\Delta F^{adh}$ is dependent on $\gamma_{cv}$, $\gamma_{hv}$, and $\gamma_{cl}$, and any change in these interfacial tensions will affect $\Delta F^{adh}$. In the absence of any protective additives, the values of $\gamma_{hv}$ is greater than that of $\gamma_{cv}$, and this results in a negative value of $\Delta F^{adh}$ (equation 4.2). The presence of Pluronic F68 or Methocel E50 lowers the $\gamma_{hv}$ of the medium, such that $\gamma_{hv} < \gamma_{cv}$, making $\Delta F^{adh}$ positive. The values of $\gamma_{cl}$ are too low to influence $\Delta F^{adh}$ greatly.

Contact angle data for a particular cell line was obtained with both HBSS and the respective culture medium without any additives. Table 4.2 shows that these values of contact angles are similar. Student's t-test was conducted in each of these cases, and no statistically significant difference was found between the readings obtained with two different test liquids. Based on this observation, the average values of the contact angles for each cell line (Table 4.2) were calculated from the combined data set obtained with both the liquids. The result proves that the $\gamma_{cv}$ is a property of the cell surface and it is not
influenced by the liquid used in measurements.

Experiments were also conducted to study the effect of the composition of growth medium on the values of \( \theta \), and thereby, on \( \gamma_{cv} \) of a particular cell line (Tables 4.2 and 4.3). There exists no statistically significant difference between the values of \( \theta \) made by HBSS on a monolayer of SF9 cells grown in a serum-containing medium (TNMFH) and a serum-free medium (SFML). It is also observed that the presence of Methocel E50 in TNMFH does not affect \( \theta \). However, the presence of Pluronic F68 in either TNMFH or SFML brought about a small change. The values of contact angles were lowered when the cells were grown in the presence of Pluronic F68. The reason of such changes is not known presently. However, one can speculate that there might be a possibility of interaction between the cell membrane and Pluronic F68. It is also possible that some of the protective agents can attach to the cell surface, and can leach out during the experiment. Leaching out of Pluronic F68 into the test liquid would definitely lower the \( \gamma_{cv} \) of the test liquid, thus lowering the contact angle.

Two different techniques (Table 4.2) have been used to measure the values of contact angles. Only anchorage dependent cells were used in the case of Wilhelmy Plate method. It was observed that in both cases (CHOK1 and N-VERO cells) the values obtained by these two methods are different. The values obtained by the ADSA-CD technique gives the equilibrium contact angle (Absolom et al., 1983, 1986; Duncan-Hewitt et al., 1989), while the values obtained with the Wilhelmy plate method gives the advancing and the receding angles as the liquid moves with respect to the cell surface.

It can be observed that the values of \( \gamma_{cv} \), using \( \theta \) obtained by the ADSA-CD technique, of the different cell lines studied were similar, though not exactly same. The values obtained for the N-VERO cells are slightly different from the other cell lines, though this difference is small. This indicates that the values of \( \gamma_{cv} \) are dependent on particular cell surface properties. The values of \( \gamma_{dl} \), as seen in Tables 4.3, 4.4 and 4.5, are
very low. The medium composition did not have any effect on $\gamma_{cl}$, (or at least does not increase), except for Pluronic F68 which slightly decreased the already low value of $\gamma_{cl}$. This corresponds to the general observation that the addition of any surface-active agent will lower, not increase the surface tension of a liquid. In addition, even if $\gamma_{cl}$ is lowered, the initial value is so low with respect to the other two interfacial tensions ($\gamma_{cv}$ and $\gamma_{dv}$) that any lowering of $\gamma_{cl}$ will result in an insignificant change in the value of $\Delta F^{\text{adh}}$.

Using the equations proposed by Neumann et al. (1974), the relative sensitivity of the values of $\gamma_{cv}$ and $\gamma_{cl}$ to contact angle, for a set value of $\gamma_{dv}$, (72.8 ergs/cm$^2$) is shown in Figure 4.4.
Figure 4.4  Plot of the relative sensitivities of $\gamma_{cv}$ and $\gamma_{cl}$ with contact angles. $\gamma_{cv}$ and $\gamma_{cl}$ are in ergs/cm$^2$, and $\theta$ is in degrees.
Since cells can be freely suspended in liquid systems, the relative hydrophobicity of cells is low. Consequently, the contact angle that a liquid can make on a cell surface will be low (<50°). All of the contact angles reported in this paper are less than 35°. As can also be observed, $\gamma_{cl}$ is relatively insensitive to changes in the contact angle in this range ($0 < \gamma_{cl} < 2.5 \text{ ergs/cm}^2$). A more noticeable change is observed with $\gamma_{cv}$, which ranges from 73 to 62 ergs/cm$^2$ for the same contact angle range (0 to 35°). However, even with $\theta$ equal to 50°, the value of $\gamma_{cv}$ is 53 ergs/cm$^2$ and $\gamma_{cd}$ is 6.5 ergs/cm$^2$.

The purpose of this sensitivity analysis was to demonstrate that even if there was a large error associated with the determination of $\theta$, the value of $\Delta F^{adh}$ changes from a negative to a positive value when protective additives, which prevent cell adhesion to gas-liquid interfaces, are present in the liquid medium. Even if there exists an error of 50% in the contact angle determinations, the above conclusions still hold. This is true for the results obtained from both the ADSA-CD technique and the Wilhelmy Plate method.

While the procedure used to measure the cell interfacial tension was based on the work of Absolom et al. (1983, 1986) and Duncan-Hewitt et al. (1989), concern was raised with respect to the viability of the cells during the measurements. To address this concern, an experiment was conducted in which the SF9 cells were prepared in the same way as required by the ADSA-CD protocol, except that instead of placing a drop of the test liquid on the monolayer of cells, the monolayer was put back into culture medium. The cell viability and appearance were determined two days after the procedure. The cell viability was determined by trypan blue exclusion viability test. After two days, in two tests 70% of the cells were viable while in one test the viability was only 5%. During these experiments and all contact angle measurements, no cell lysis was observed. While this procedure does have some effect on the cells, this effect is not catastrophic and we believe that the results of the contact angle are representative of the properties of a normal cell membrane.
4.9 Conclusions

The thermodynamic equation was used to explain cell adhesion to gas-liquid interfaces. Several experimental techniques on different cell lines were used to verify this relationship. It also demonstrates the non-specificity of cell-gas adhesion. An implication of this relationship is that cell-gas adhesion is not thermodynamically favorable if $\Delta F^{adh}$ is positive, and this was confirmed experimentally. The most practical method of increasing $\Delta F^{adh}$ is by the lowering of $\gamma_v$ since: (1) $\gamma_c$ for a particular cell line is virtually unaffected by the composition of the liquid medium, and (2) $\gamma_d$ is so small compared to the other two values that a change in its value will not have a significant effect on $\Delta F^{adh}$. Due to the inherent variability in the experimental techniques used to calculate $\gamma_c$, $\gamma_d$, $\gamma_v$ should be significantly lower than $\gamma_c$ to prevent cell adhesion to gas-liquid interfaces.

4.10 Appendix

One of the first set of equations for determination of $\gamma_d$ and $\gamma_{sv}$, proposed by Owen and Wendt (1969), was motivated by adhesion of liquids to polymeric surfaces. Their work was based on earlier work by Fowkes (1964) who considered that the molecules at a particular interface are subjected to a resultant force field. This field is composed of components arising from the bulk attractive forces in each phase and the London dispersion forces operating across the interface itself. Owen and Wendt (1969) also considered the hydrogen bonding (h) forces as contributing factors to the total free energy at the surfaces in addition to the dispersive (d) forces. Considering these two forces, the interfacial tension between any two phases, 1 and 2, can be expressed as the summation of its two components: dispersive forces and hydrogen bonding forces. $\gamma_{12}$ can be expressed as:
\[ \gamma_{12} = \gamma_{12}^h + \gamma_{12}^d \] (4.11).

According to Owen and Wendt (1969), the value of \( \gamma_{st} \) is expressed by:

\[ \gamma_{st} = \gamma_{sv} + \gamma_{sv} - 2\sqrt{\gamma_{sv}\gamma_{sv}^d} - 2\sqrt{\gamma_{sv}\gamma_{sv}^h} \] (4.12).

The value of \( \gamma_{st} \) in the above equation can be eliminated by using Young's equation (equation 4.4) which will introduce the contact angle, \( \theta \), as:

\[ \gamma_{tv} (1 + \cos \theta) = 2(\sqrt{\gamma_{tv}\gamma_{tv}^d} + \sqrt{\gamma_{tv}\gamma_{tv}^h}) \] (4.13)

In equation (4.12), \( \theta \) and \( \gamma_{tv} \) can be determined experimentally, and from these values \( \gamma_{sv} \) can be calculated. Suitable assumptions regarding the values of \( \gamma_{12}^h \) and \( \gamma_{12}^d \) can be made depending on the polarity of the system.

Neumann et al. (1974) proposed another equation of state to provide a relationship between the three interfacial tensions and the contact angle in a solid-liquid-vapor system. It was shown from thermodynamic considerations, by Ward and Neumann (1974), that the interfacial tensions of the three interacting phases are interrelated. Neumann et al. (1974) used an interaction parameter, \( \Phi \), known as Good's interaction parameter, for relating the different interfacial tensions, and this parameter is expressed as:

\[ \Phi = \frac{\gamma_{tv} + \gamma_{tv} - \gamma_{st}}{2\sqrt{\gamma_{sv}\gamma_{tv}}} \] (4.14)
Neumann et al. (1974) assumed that \( \Phi \) is a function of \( \gamma_{sl} \) only, assuming \( \gamma_{sv} \) to be constant for a particular system. In addition, various plots of \( \gamma_c \cos \theta \) versus \( \gamma_{iv} \) for different systems were made and it was concluded that \( \gamma_{sl} \) has its minimum value when the contact angle is zero (\( \gamma_{sl}^* = 0 \)). Also, at this condition, \( \gamma_{sv} \) is equal to \( \gamma_{iv}^* \). It should be noted at this stage that \( \gamma_{12}^* \) is the interfacial tension when the contact angle is equal to zero, indicated by a 45° line on a plot (Neumann et al., 1974) of \( \gamma_c \cos \theta \) versus \( \gamma_{iv} \).

Considering these considerations, the values of \( \gamma_{sv} \) were determined from the plots. Values of \( \gamma_{sl} \) were then calculated from equation (4.4) using experimentally determined values of \( \gamma_{iv} \) and \( \cos \theta \), and the calculated value of \( \gamma_{sv} \). Subsequently, the values of \( \Phi \) were determined from the different values of \( \gamma_{sv}, \gamma_{sl}, \gamma_{iv} \) and \( \cos \theta \). On plotting \( \Phi \) as a function of \( \gamma_{sv} \), for different systems, a straight line of the form:

\[
\Phi = -a \gamma_{sv} + b
\]  

(4.15)

was obtained with \( a = 0.00775 \) and \( b = 1.0 \). Combining equations (4.14) and (4.15), \( \gamma_{sl} \) can be expressed as:

\[
\gamma_{sl} = \frac{[\gamma_{sv}^2 - (\gamma_{iv})^2]^{\frac{1}{2}}}{1 - 0.015(\gamma_{sv} \gamma_{iv})^{\frac{1}{2}}}
\]

(4.16)

\( \gamma_{sl} \) can be eliminated from the above equation by combining equation (4.16) with Young's equation as:

\[
\cos \theta = \frac{(0.015 \gamma_{sv} - 2.0) \gamma_{sv} \gamma_{iv} + \gamma_{iv}}{\gamma_{iv} [0.015 \gamma_{sv} \gamma_{iv} - 1]}
\]

(4.17)
Neumann *et al.* (1974) noticed that on plotting \( \cos \theta \), obtained from equation (4.17), as a function of \( \gamma_{sv} \), for different \( \gamma_{iv} \), there occurs a discontinuity in the function for high values of \( \gamma_{sv} \), such as water. A FORTRAN program was written by Neumann *et al.* (1974) to circumvent this problem.

In 1982, Gerson developed another set of empirical equations, which is actually an improvement on the set of equations (equations 4.16 and 4.17) proposed by Neumann *et al.* (1974). The equation proposed by Gerson (1982) provides a continuous function that is applicable for both high and low energy solids. The main inspiration in obtaining a continuous function came from the concept that the interaction parameter, \( \Phi \), is a function of both \( \gamma_{sl} \) and \( \gamma_{sv} \), and is of the form:

\[
\Phi = \exp[\gamma_{sl}(a \gamma_{sv} + b)]
\]

(4.18)

instead of only \( \gamma_{sl} \), as considered by Neumann *et al.* (1974). In this case there are two sets of equations: the first one for the determination of \( \gamma_{sv} \) from experimentally determined values of \( \gamma_{iv} \) and \( \theta \):

\[
\frac{\gamma_{iv} \, (\cos \theta + 1)}{2 \gamma_{iv} \gamma_{sv}} \exp[(\gamma_{sv} - \gamma_{iv} \cos \theta)(a \gamma_{sv} + b)] = 0
\]

(4.19),

and the second equation allows calculation of \( \gamma_{sl} \) from \( \gamma_{iv} \) and \( \gamma_{sv} \):

\[
\frac{\gamma_{iv} + \gamma_{sv} - \gamma_{sl}}{2 \gamma_{iv} \gamma_{sv}} \exp(\gamma_{sl}(a \gamma_{sv} + b)) = 0
\]

(4.20)

Equation (4.20) was obtained by combining equation (4.18) with equation (4.14), and equation (4.19) was obtained by using Young's equation (equation 4.4) to replace \( \gamma_{sl} \) in
equation (4.20). The above two equations can be solved numerically with the best-fit values of the parameters: $a = 6.5 \pm 1.0 \times 10^{-5}$ and $b = -0.010 \pm 0.001$, which were provided by the author (Gerson, 1982).
CHAPTER V
EFFECT OF PROTECTIVE ADDITIVES ON THE LIPID COMPOSITION OF CELLS

5.1 Summary

Large-scale insect and animal cell cultures can be propagated in bioreactors. In order to provide a homogeneous environment in the bioreactor, and also to supply adequate amount of oxygen to the growing cells, these bioreactors are often equipped with spargers. However, air sparging causes cell damage, and this reduces the productivity of the system. Researchers have shown that cell damage in sparged bioreactors is mainly due to cell-bubble interactions. Research is conducted to reduce the degree of cell damage in bioreactors, and one of the solutions to the problem of cell damage is the addition of protective agents to the culture medium. These additives prevent cell damage mainly by reducing the cell-gas adhesion. From thermodynamics, the feasibility of the process of cell-gas adhesion is dictated by the magnitude of $\Delta F^{\text{adh}}$, which is the change in free energy for the process, and $\Delta F^{\text{adh}}$ is dependent on the interfacial tensions between the different phases (cell, liquid, and vapor phases) present in sparged bioreactor. Any changes in the different interfacial tensions will change the magnitude of $\Delta F^{\text{adh}}$ such that the process can go from a feasible state to an unfavorable state. It has been observed earlier that the presence of the protective additives in the liquid medium can influence the values of interfacial tensions between the different phases, especially between the liquid and the vapor phases. However, it is also possible that the interfacial tensions between the cell and the vapor phase can be affected by the presence of these additives. Evidence, both direct and indirect, exists that there occurs
some interaction between the cell membrane and the components in the liquid medium. Considering these studies, preliminary investigation has been conducted to determine the existence of interactions between protective additives (Pluronic F68 and Methocel E50) present in the liquid medium and cells by using chromatographic techniques. Thin-layer chromatography (TLC) has been chosen as the method to conduct such studies.

5.2 Introduction

Advances in recombinant DNA technology has made possible the production of foreign proteins, of great commercial value, in insect and animal cells. Commercialization of these products has also led to the development of large-scale production systems for insect and animal cells. Though these cells are preferred over the bacterial cells, they are shear-sensitive in nature and are easily damaged by high hydrodynamic shear stresses present in the bioreactor. In order to overcome this problem, different types of bioreactors have been designed. The formulations of the media can also be changed to make the cells more resistant and this can be accomplished by the addition of various polymeric additives, known as protective agents, such as pluronics, derivatives of celluloses (methylcelluloses, hydroxymethylcelluloses), serum, hydroxyethyl starch (HES), polyvinyl alcohol (PVA), polyethylene glycol (PEG), some dextrans, and others.

Due to the high oxygen demand of the insect and the animal cells, sparging is an essential feature in the bioreactors used for growing them. However, air sparging leads to bubble entrainment, and cell-bubble interactions leads to extensive cell damage in bioreactors (Kilburn et al., 1968; Tramper et al., 1986a; Handa-Corrigan et al., 1987, 1989; Kunas et al., 1990a; Bavarian et al., 1991; Chalmers et al., 1991). Chattopadhyay et al. (1995a) have shown cell damage is directly proportional to cell-gas adhesion and elimination of cell-gas adhesion will definitely reduce the amount of cell damage taking place in a particular sparged culture. They have also shown that the presence of certain
additives, such as Pluronic F68 and Methocel E50 and E4M (hydroxypropyl methylcelluloses), in the culture medium reduce the degree of cell-gas adhesion. These additives have also been found to be very effective in protecting the cells from hydrodynamic damage, and this was shown by the decrease in the number of cells killed per bubble ruptured. Based on these observations made, Chattopadhyay et al. (1995a) have concluded that one of the ways of preventing cell damage in bioreactors is to prevent the cells from coming in contact with the bubble. The effectiveness of the protective additives depends on their ability to prevent the cells from adhering to the bubble surface. On measuring the liquid-vapor interfacial tension (γ_{lv}) of the liquid medium in the presence of different protective additives, it was found that the most effective protective agent was the one that reduced the γ_{lv} of the liquid medium within the life-time of a bubble in a bioreactor. This also shows the relevance of dynamic γ_{lv} over the equilibrium value in determining the effectiveness of the protective additives. Chattopadhyay et al. (1995b) have also measured the remaining two interfacial tensions (cell-vapor interfacial tension, γ_{cv}, and cell-liquid interfacial tension, γ_{cl}). Thermodynamic study (Chattopadhyay et al., 1995b) have shown that the feasibility of the process of cell-gas adhesion is dependent on the change in the free energy (ΔF^{adh}), which can be expressed as:

$$\Delta F^{adh} = \gamma_{cv} - \gamma_{lv} - \gamma_{cl}$$  (5.1)

The ΔF^{adh} is dependent on the different interfacial tensions involved in the system. From the results obtained (Chattopadhyay et al., 1995b), speculations were made that there exists a possibility of interaction between the protective additives (especially Pluronic F68) and the cells, and this has influenced the values of contact angles made by the test liquid on a monolayer of cells. Such interactions were observed when *Spodoptera*
frugiperda (SF9) cells were grown in the presence of Pluronic F68, either in TNMFH medium or in SFML medium. Further speculations can be made about the type of interaction between Pluronic F68 and SF9 cells. The interaction can be both physical and chemical in nature. A physical interaction can be due to adsorption Pluronic F68 on the cell surface. The chemical interaction can be as formation of chemical bonds between Pluronic F68 and components (lipids and/or protein molecules) of the cell membrane. So, understanding the chemistry of interaction between the cells and the protective additives is important in designing a system that would reduce cell damage in bioreactors.

5.3 Evidence Of Interactions Between Cells And Environment

Studies conducted in order to determine the possibility of an interaction between the cell membrane and its growth environment can be broadly divided into two categories: indirect evidence, and direct evidence. The first group have studied the change in some of the properties of the cell membranes, such as increase in resistance to shear, in order to account for any interaction between the additives present in the growth medium and the cell membrane. The second group have actually studied the cell membrane to detect changes in the profile of the composition that might be a result of the presence of the additional component in the growth medium. The protective agents are added to the growth medium when the cells are cultured, and if the cells can interact with the components of the growth medium and incorporate them in their membrane, there also exists the possibility of interaction between the protective agents and the cell membrane.

Interaction of the cell membrane with various additives like serum (Ramirez et al., 1992), and Pluronic F68 (Kilburn et al., 1968; Mizrahi, 1984; Murhammer et al. 1990a, Goldblum et al., 1990) with the cell membrane has already been found. Serum, which is added to the culture medium to enhance cell growth and acts mainly as a nutrient, was
also found to act as a protective agent. It has been shown by Ramirez and Mutharasan (1990, 1992) that the nutritional and the protective aspects of serum are independent of each other. They evaluated the plasma membrane fluidity (PMF) of the cells and correlated it to the shear sensitivity of a particular cell line. The PMF of intact hybridomas was measured by using the steady-state fluorescence polarization technique, and a cationic fluorescent probe was used to label the cells. Ramirez and Mutharasan (1990) studied the effect of a cholesterol enriched medium on cells (HB-32 hybridomas), and they observed that the PMF of the cells decreased and this is directly due to the increase in cholesterol content of the cell membrane. Cholesterol is an essential constituent in the plasma membrane of the animal cells, and it has an important role in the regulation of PMF. The decrease in PMF was demonstrated by the increase in the resistance of the cells against laminar shear stress. This showed that cholesterol was transferred from the medium containing serum to the membrane. In 1992, a mechanism for the protective effect of serum was proposed by Ramirez and Mutharasan. According to them, transfer of cholesterol or analogous compounds from the medium to the membrane modulate the PMF. They also suggested that the direct measurement of the lipid composition of the cell membrane as a function of the serum concentration in the culture medium would provide an insight into the protective mechanism of serum. Kunas and Papoutsakis (1989, 1990b) showed that hybridoma CRL 8018 cells became more resilient within a very short period after serum was added to the growth medium. This led them to conclude that the protective action was not just nutritional, as the time period is not sufficient for the cells to metabolize the additives. It is probable that the cells became more shear-resistant due to some interaction between the serum, present in the growth medium, and the cell membrane. Incorporation of the serum molecules in the membrane may have resulted in the increased strength.
There exists further evidences regarding incorporation of the protective additives in the cell membrane and in all of these cases, the inference is based on the increase in shear resistivity of the cells in the presence of these additives. Evidence of adsorption of the protective additives on to the cell membrane was provided by Murhammer et al. (1990b) when they studied the effect of Pluronic F68 on sparged cultures of the insect cell line, SF9. Similar proposals were also put forward by Kilburn and Webb (1968) and Mizrahi (1984). Mizrahi (1984) found that the uptake of glucose by the cells decreased in the presence of carboxymethyl cellulose or hydroxyethyl starch, and this led him to conclude that these polymers protect the cells by forming a protective layer. Evidence of interaction between Pluronic F68 and the cell membrane was also provided by Smith et al., 1987, Clarke et al., 1992, and Zhang et al., 1992. In all of these cases, there occurred an increase in the shear-resistivity of the cells. In 1990, Goldblum et al. studied the effect of protective additives on two insect cell lines, TN368 and SF9, and these cells were found to become more resistant to laminar shear stress in the presence of protective additives, such as Pluronic F68 and Methocels. They also found that the amount of protection from laminar shear stress was the same regardless of whether the cells were grown or re-suspended in medium containing additives. From the experimental results obtained, they concluded that the polymeric additives were adsorbed on to the cell membrane and the adsorbed layer provided protection against the laminar shear stresses.

The influence of the environment on the lipid composition of the cell membrane has been shown by a number of research groups. A number of researchers have studied the cell membranes after the cells were grown in medium containing certain additional compounds. Studies were also conducted with protective agents, such as dextran and Pluronic F68. Studies done with erythrocytes treated with high molecular weight molecules of dextran showed a tight association of the dextran molecules with the cell membrane under electron microscopes (Cudd et al., 1989). Joshi et al. (1989) showed
that digitonin, which is a surfactant with cyclic hydrophobic moiety, increases the permeability of lactose-fermenting yeast to lactose. The effect was visible within 30 minutes of addition of the surfactant to the yeast cells. The mechanism of permeabilization could be due to the formation of a complex with the cholesterol present in the cell membrane and this renders the membrane leaky. Scallen et al. (1969) have shown that the presence of digitonin in the medium retains cholesterol in the membrane by the formation of the cholesterol-digitonin complex.

King et al. (1991) also showed that the permeabilization of yeast cells can be brought about by the presence of a surfactant in solution. Pluronic F68 was found to increase the rate of fluorescein diacetate uptake into the yeast cells, and it was proposed that this increase in rate is due to the increase in membrane permeability, which is brought about by the addition of Pluronic F68 to the culture medium. Experimental results obtained by them showed that Pluronic F68 directly affects membrane functions. It has been found by cryo-SEM analysis that Pluronic-treated yeast cells revealed a coating action, and this coating action is not merely physical, but involves direct interactions with the cells leading to alterations in membrane function.

Fonvieille et al. (1992) evaluated the effect of keratin on the lipid composition of Scopulariopsis brevicaulis, an organism responsible for certain human fungal infections. The most prevalent form of keratin, which is a fibrous protein, is composed mainly of polypeptide chains in the α-helical conformation (α-keratin), or β-sheets (β-keratin). The α-helix conformation is rich in proline, glycine, and serine residues. The keratin in the culture medium serves as a nitrogen source. This organism was cultured in medium with and without keratin. They found that the lipid content was lower in the presence of keratin. There was also a difference in the unsaturated fatty acid content in the presence of keratin. This shows that the presence of keratin as nitrogen source in the culture medium has affected the lipid content and the fatty acid composition of the membrane.
and this might be the reason for morphological and ultrastructural alterations observed in the presence of keratin. Rolph et al. (1991) showed the effect of plant growth regulator paclobutrazol on suspension cultures of celery, and it was shown by them that they alter the cell membrane sterol composition.

The effect of the growth environment of the cells on the lipid composition was shown by other studies. Huflejt et al. (1990) showed that the salinity of the culture medium greatly influences the lipid composition of the membrane of Synechococcus 6311. On transferring the cells from a low salt content culture to a high salt content culture, it was observed that there was a rapid decrease in palmitoleic (C16:1) acid content, along with an increase in the C18:1 acids. The changes in the fatty acid content were observed within the first hour of transfer, and this indicates physical interaction between the lipids in the cell membrane and the salt in the medium. Thiemann et al. (1991) showed that with increasing salinity there occurs an increase in phosphatidylglycerol, and a decrease in phosphatidylethanolamine. Results from these studies show that salt in the culture medium alters the lipid composition of the membrane, and this change affects the membrane permeability and other properties. The change in lipid content due to salinity may be due to charge interactions. It has been observed that there occurs an increase in the polarity of membrane lipids in higher salt concentrations.

Nozawa (1980) have reported the effects of different compounds in the culture medium on modification of lipid composition of Tetrahymena. Supplementation of various compounds, which are not metabolized by the cells, in the culture medium can be incorporated in the cell membrane. Supplementation of ergosterol in the culture medium for Tetrahymena have shown to incorporate ergosterol in the membrane in exchange of tetrahymanol, a sterol-like membrane component. This replacement causes significant changes in the fatty acid composition of the membrane phospholipids. It has also been
seen that the polar head group composition is also modified due to the ergosterol substitution. The author has shown that when compounds such as hexadecyl glycerol, fatty acids, and isovalerate are present in the culture medium, they are incorporated in the cell membrane. As a result of this incorporation, significant changes in the fatty acid composition and lipid content has been observed. Modification in the lipid profile was also observed when the cells were treated with phenethyl alcohol (PEA). Change in the lipid profile also altered the physical properties of the cell membrane.

Thus, the findings of various researchers show that culture medium supplemented with various compounds have the capability of incorporating these compounds in the cell membrane. This incorporation can change the lipid composition of the membrane, and altered lipid composition can alter the physical properties of the cell membrane.

5.3.1 Non-Specificity Of Interactions Between Additives And Lipid Bilayer

For the present study, SF9 cells grown under different conditions have been studied to show the effect of the additives on the cells, and determine whether the interaction is guided by specific forces (interactions with specific receptors for the additive) or non-specific forces. Murhammer et al. (1990b) have shown that hydrophilic-lipophilic balance (HLB) of the additives is responsible for their protective action, and compounds of similar structural features have shown similar action. This evidence points in the direction of non-specificity. It has also been seen that some of these additives have been used with a number of cell lines, such as insects, animals, and plants. It has also been observed that a particular cell line can be protected by a variety of additives. These observations do not indicate a specific receptor for the additives. Moreover, the lipids are linked to other cellular components by non-specific forces, such as hydrogen bonding, van der Waals' forces and by ionic bonds. Most of the additives are also amphipathic in nature as the lipids, and there is a possibility that they will interact with the lipids through
these non-specific forces. However, further evidence is required to substantiate the idea that the protective effect shown by the additives is non-specific in nature.

5.4 Lipids In Biological Membranes Of Insect And Animal Cells

Mammalian cells are surrounded by a phospholipid bilayer (plasma membrane) embedded with enzymes and structural proteins, which mediate communication between the cell and the environment. Both the insect and the animal cells lack an outer cell wall and as a result are highly sensitive to environmental stimuli, such as osmotic changes, hydrodynamic forces, pH and nutrient changes.

In animal tissues, the structural lipid components, such as phospholipids, tend to be rather constant. On the other hand, the proportions of the simple lipids, especially the triacylglycerols, can vary greatly according to the dietary or physiological state of the animal. The fatty acid composition of each lipid in a tissue is frequently distinctive and can vary markedly between species.

Phospholipids are those that contain phosphorus, and they are soluble in non-polar solvents. The two main groups of phospholipids are phosphoglycerides and phosphosphingolipids. The two most important groups of phospholipids found in extracts from insects are phosphatidylcholine and phosphatidylethanolamine (Bridges, 1983). However, phosphatidyl-serine, -inositol, and -glycerol, phosphatidic acid and polyglycerophosphatides have also been found in varying quantities in different insects. Interestingly, it has been noted that the insect phospholipids can be altered by changing the diet of the insects (phospholipid bases can be replaced by their analogs). Most of the initial studies on lipid composition in insects were done on extracts of the whole insect. However, studies conducted with extracts from different tissues from an insect, in some cases, gave a different lipid profile than that obtained from whole extracts.
The fatty acid composition in insects, in general, has been reported by Stanley-Samuelson et al. (1988). The whole-insect fatty acid composition of all insect orders is fairly similar, in a qualitative way. The profile seemed to include about eight components, most of them saturated and monounsaturated fatty acids plus two polyunsaturated fatty acids - C18:2 and C18:3. However, there are exceptions to this generalization. In a quantitative way, generalization is virtually impossible, as the fatty acid composition differs with different stages in development and with different diets. There are also intertissue differences within an organism and differences due to environmental effects.

Grapes et al. (1989) have carried out a detailed fatty acid and lipid analysis of the house cricket, *Acheta Domesticus*. The result obtained shows the typical fatty acid and lipid composition of an insect. However, for our present studies we will be concentrating on the extract from ovaries, since SF9 cells were isolated from ovaries of insects. It has been found that the fatty acid composition of the ovaries of the mature female adult insect is linoleate (C18:2), palmitate (C16:0), oleate (C18:1), and stearate (C18:0). Smaller amounts of other acids are also found, like, laurate (C12:0), myristate (C14:0), palmitoleate (C16:1), linolenate (C18:3), and arachidate (C20:0). Triacylglycerols are predominant in ovarian, egg and fat-body lipid extracts. High levels of lipid are found in these tissues. The major phospholipids present in all tissue extracts were phosphatidylcholine and phosphatidylethanolamine. Cholesterol is the major sterol present.

Grau et al. (1971) reported the fatty acid composition of TN368 cells. They found that six fatty acids were consistently detected in TN368: palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3). Louloudes et al. (1973) have studied the fatty acid profiles of *Spodoptera frugiperda* (IPRL-21) cells when these cells were cultivated in IPL-1 medium containing 5.6% fetal
bovine serum. At least 23 fatty acids were identified from the media and/or from the cells. Gas chromatography was used for this purpose. Seven of the 23 fatty acids (palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidonic acids (C20:4)) comprised 92% of the total fatty acid content.

With the recent advances made in the area of cell cultures with insect and animal cell lines, and the increasing requirement of eliminating serum from the growth medium, studies have been conducted to estimate the role of lipids in the culture medium. The presence of serum in the culture medium is the sources of fatty acids needed for cell growth. Most of the serum-free media formulations have added lipids to enhance cell growth. The added lipids act as exogenous sources of sterols and fatty acids.

5.5 Materials Used

Two different insect cell-lines were used for the study: *Spodoptera frugiperda* (SF9) cells, and *Trichoplusia ni* (TN368) cells. Both these cell-lines were subcultured in six different media/additive combinations: (i) serum-containing medium (TNMFH) with 10% v/v fetal bovine serum (FBS) without any additives, (ii) TNMFH with 0.1% w/v Pluronic F68, (iii) TNMFH with 0.3% w/v Methocel E50, (iv) serum-free media supplemented with lipids (SFML), (v) SFML with 0.1% w/v Pluronic F68, and (vi) SFML with 0.3% w/v Methocel E50. Hank's balanced salt solution (HBSS) was used to re-suspend the cell pellet after the growth medium was removed. Thin chromatographic plates (Alltech) were used for the study. The solvents used were at least of reagent or chromatographic grade purity. All the experiments were conducted in glassware cleaned with a proper detergent followed by acid solution. The cleaned glassware was then washed with chloroform-methanol (2:1 v/v) solution prior to use.
5.6 Experimental Techniques

Researchers have provided evidences regarding the interaction between the cell membrane and the additional components present in the liquid growth medium. Studies have also been conducted with protective agents (Pluronic F68, dextran) and the results have pointed towards the association between the additives and the cell membrane. But, till today, no analysis of the lipid bilayer of the membrane was conducted to show how the protective additives bind to the cell membrane. For our present study, we intend to show the effect of protective additives on the lipid composition of the cells, as any change will be a direct proof of interaction between the interaction between the additives and the cell membrane. This will also help in understanding the chemistry of such interactions. Thin-layer chromatography (TLC) is the method of our choice for lipid analysis because of its simplicity, inexpensiveness, rapidity, sensitivity, and selectivity.

The cells were grown in the different media, with or without protective additives, in T-flasks. The cells were tested after they have reached their exponential growth phase, and the concentration of cells in the T-flasks has reached > 1.0 x 10^6 cells/mL. The cells were resuspended and transferred to centrifuge tubes. The cell suspension was centrifuged at 2000 rpm for 5 minutes. The cell pellet formed after centrifugation was resuspended in HBSS. In this way, the culture medium was eliminated from the experiments, so that the lipid profile obtained on the TLC plates will be from the cells only, and not from the medium.

The first step in any lipid analysis of the cell is the isolation of lipids from tissues by extraction with organic solvents, and the removal of non-lipid contaminants from this extract. Special precautions were taken to ensure that there was no contamination and recovery was complete. The solvent mixture used for extraction of lipids from the insect cells (SF9) was chloroform-methanol mixture. The criteria for the choice of the solvent system were that they should be sufficiently polar to remove all lipids from their
association with cell components, but should not react chemically with these lipids. At the same time, the polarity of the solvents should not be very high so that non-polar simple lipids do not dissolve, as we want to observe the complete lipid profile. The solvent system chosen satisfies these criteria.

Folch extraction method (Kates, 1986) was used for the experiment. This extraction gives 95 to 99 percent recovery of lipids. This method gives excellent recoveries for neutral lipids, diacylglycerophospholipids and most of the sphingolipids. The major merits of Folch's method are that much smaller amounts of solvents are required and that less non-lipid material is retained in the extract. The lipid extract was finally concentrated using nitrogen blow-down procedures. Though the separation is not very complete, it is simple to use when there are a number of samples, as it is less time-consuming.

5.7 Results

Figures 5.1a to 5.1e show the results obtained from conducting TLC analysis on the SF9 cells grown in different medium/additive combination. By comparing the chromatograms and excluding similar spots on the TLC plates, the differences in the lipid profiles were studied.
Figure 5.1 Chromatograms for SF9 cells: a - SF9 cells grown in TNMFH; b - SF9 cells grown in TNMFH plus 0.1% Pluronic F68; c - SF9 cells grown in TNMFH plus 0.3% Methocel E50; d - SF9 cells grown in SFML; e - SF9 cells grown in SFML plus 0.1% Pluronic F68.
5.8 Discussion

The main purpose of this set of experiment was to check the possibility of any change brought about by the presence of the protective additives in the cell membrane. From an earlier study (Chattopadhyay et al., 1995a), it has been seen that the different additives have shown different protective action. Chattopadhyay et al. (1995b) have shown that when the cells were grown in the presence of Pluronic F68, the value of $\gamma_{cv}$ (interfacial tension between the cell and the vapor phase) of the SF9 cells differs slightly from those which were grown without any additives or in the presence of Methocel E50. It was also speculated that there exists a possibility of interaction between Pluronic F68 and the lipid components of the cell membrane. However, from the results obtained it is seen that apparently there is no change in the lipid profile of the cells in the presence of Pluronic F68. While analyzing the results, it should be kept in mind that the lipid analysis was conducted with a single solvent system (chloroform-methanol-water).

Definite conclusions can not be drawn regarding the possibility of change in the lipid composition of the cells in the presence of the protective additives. In order to obtain a more conclusive result, different solvent systems should be tested. Moreover, the protective additives (Pluronic F68 and Methocel E50) are polar in nature, as they are soluble in polar solvents. There exists a possibility that these compounds and the compounds, if any, formed with the association with the protective additives will partition into the aqueous phase. Suitable protocols need to be devised to examine the components present in the aqueous phase.

Knowledge of interactions between the additives and the membrane is required as change in the lipid profile of the cell membrane will change the physical properties of the membrane, and as a result, can make the cells more shear-resistant.
CHAPTER VI

SUMMARY

Both insect and animal cell cultures are becoming recognized as important technology for the production of biologicals, including recombinant proteins and biopesticides. The lepidopteran-baculovirus expression vector system for insect cells is by far the main driving force behind insect cell culture engineering. The standard cell line in most widespread use is SF9 from *Spodoptera frugiperda*, and the most common vector for protein expression consists of genetically engineered *Autographa californica* nuclear polyhedrosis virus (AcNPV).

Though insect and animal cells are used in a number of large-scale processes, their shear sensitivity was a bottle-neck towards the scaling up process. One of the solutions to this problem is protecting the cells in the bioreactors by adding protective agents in the culture medium. In this present study, efforts have been made to identify protective additives that will prevent the cells from damage by hydrodynamic forces in the bioreactors. Efforts have also been made to understand the mechanism of the protective action of these additives.

Different protective additives have been studied, and they are: Pluronic F68, Methocels A4M, E4M and E50, polyvinyl alcohol (PVA), polyethylene glycol (PEG) of molecular weights 400, 1000, 4000, and dextran. The effectiveness of these protective additives was determined from the number of cells killed per bubble ruptured in the presence of these additives. Figure 6.1 shows the relative effectiveness of the different additives studied.
Figure 6.1 Relative protective efficiencies of the different protective additives studied.
The results show that Pluronic F68 and Methocels E50 and E4M are the most effective protective agents. It should be kept in mind that these experiments were carried out the insect cell line, SF9, which were grown in different media/additive combinations.

The addition of the protective additives affects the value of $\gamma_{LV}$ of the liquid medium. Figures 6.2 and 6.3 show the values of $\gamma_{LV}$ obtained by different techniques for different media/additive combinations. As mentioned earlier, the dynamic value of $\gamma_{LV}$ (obtained by Maximum Bubble Pressure method) is more relevant than the equilibrium value (obtained by Wilhelmy Plate method), as the time required for dynamic measurements is equivalent to the life-time of a bubble in a typical bioreactor.
Figure 6.2  Liquid/vapor interfacial tensions of TNM-FH with/without additives obtained by Maximum Bubble Pressure method and Wilhelmy Plate method.
Figure 6.3  Liquid/vapor interfacial tensions of SFML and HBSS with/without additives obtained by Maximum Bubble Pressure method.
Comparing the results shown in Figures 6.1, 6.2, and 6.3, it can be seen that the most effective protective additives were those that were able to lower the value of $\gamma_{LV}$ under dynamic conditions. Pluronic F68 and Methocel E50 were able to meet these criteria. Though it was not possible to measure the $\gamma_{LV}$ in the presence of Methocel E4M, it is known to be surface-active in nature, and will behave similarly as that of Methocel E50.

It has also been observed that the presence of these protective additives in the culture medium prevents cell-gas adhesion. Pluronic F68 and Methocels E50 and E4M were found to be the most effective protective additives in reducing cell-gas adhesion. From these observations, it is obvious that lowering of $\gamma_{LV}$ is required to prevent cell-gas adhesion, and reduction in cell-gas adhesion is necessary to prevent cell damage from bubble rupture.

From thermodynamics, a relationship has been proposed to explain the phenomenon of cell-gas adhesion from the change in free energy ($\Delta F^{\text{adh}}$) for the process. The $\Delta F^{\text{adh}}$ is dependent on the different interfacial tensions involved in the system. Therefore, it can be stated that the protection can be brought about by changing the physical properties at the cell-liquid-vapor interface. It has been shown thermodynamically that changes in the difference between $g_{CV}$ and the summation of $g_{LV}$ and $g_{CL}$ from a negative value to a positive value will affect the feasibility of the process. A positive $\Delta F^{\text{adh}}$ will make the process thermodynamically unfavorable. The thermodynamic relationship also demonstrates that the primary protective action of the polymeric additives is non-specific in nature. Further proof of this non-specificity was shown when the conclusions drawn from the results obtained with the insect cell line was equally applicable to animal cell lines.
The presence of the protective additives brings about these changes, and the most noticeable change is the decrease in the liquid-vapor interfacial tension ($\gamma_{lv}$) of the medium. The possibility of the change in the other two interfacial tensions ($\gamma_{cl}$ and $\gamma_{cv}$) due to the presence of the protective additives has also been investigated. The values of $\gamma_{cl}$ were very low (< 1.5 ergs/cm$^2$), compared to the values of $\gamma_{cv}$ and $\gamma_{lv}$. Therefore, any change in $\gamma_{cl}$ will not affect $\Delta F_{adh}$ considerably. In any case, the presence of the protective additives, which are surface-active in nature, will decrease $\gamma_{cl}$ further. The values of $\gamma_{cv}$ are in the same range as that of $\gamma_{lv}$. Among the medium/additive combinations studied, only the presence of Pluronic F68 affected the value of $\gamma_{cv}$. However, the $\gamma_{cv}$ of the cell line studied remained the same when the cells were grown in different medium without additives (serum-containing medium and serum-free medium), and medium containing Methocel E50. Therefore, reduction in $\gamma_{lv}$ is the most feasible method of changing $\Delta F_{adh}$ from a negative value to a positive value. The values of $\gamma_{cv}$ and $\gamma_{cl}$ were determined using different semi-empirical equations, and the results obtained from these semi-empirical equations are very similar. In all the cases with medium containing no additive, $\Delta F_{adh}$, as calculated from the thermodynamic relation is negative in value, as $\gamma_{lv}$ is greater than $\gamma_{cv}$. This means that cell adhesion to the air-medium interface would take place spontaneously, which corroborates with experimental findings in medium with no additives. A positive $\Delta F_{adh}$ indicates that cell-gas adhesion will not occur and this also corroborates with the experimental results.

A 3-D plot of the thermodynamic equation for the cell-line SF9 is shown in Figure 6.4.
Figure 6.4  Plot of change in free energy with interfacial tensions for SF9.
The test liquid used for measuring $\gamma_{iv}$ was TNMFH, and the value of $\gamma_{cv}$ was obtained using the equations proposed by Neumann et al. (1974). Considering that the value of $\gamma_{cv}$ for a particular cell-line remains constant irrespective of the liquid used to determine it, a 3-dimensional graph can be plotted with $\gamma_{iv}$ and $\gamma_{cv}$ as x and y axes respectively, and $\Delta F^{adh}$ as the z-axis. At $\gamma_{iv}$ equal to 69.4 (for TNMFH) ergs/cm$^2$, the $\Delta F^{adh}$ is negative. This means that at these values of $\gamma_{iv}$, cells will adhere to the air-medium interface, and this corroborates with experimental findings. As $\gamma_{iv}$ is lowered, the value of $\Delta F^{adh}$ increases, and at a certain value of $\gamma_{iv}$, $\Delta F^{adh}$ becomes positive, and this means that at this region cell-gas adhesion will not occur. This shows the possibility of reducing the cell-gas adhesion by the process of reducing the $\gamma_{iv}$ of the culture medium. As most of the protective additives are surface-active agents, there exists a possibility of making the process of cell adhesion to the air-medium interface non-spontaneous (i.e. $\Delta F^{adh}$ is positive), by the addition of protective additives.

The hydrophobicity of the cell surface dictates the degree of adhesion of the cells to the air-medium interface in bioreactor. Measurements of the degree of hydrophobicity can be done by measurements of contact angles on the monolayer of cells, and results showed that for different cell lines studied, the value of $\theta$ is low, indicating low hydrophobicity of the cell surface. The $\theta$ varied from 7° to 34° with $\theta$ for most of the cells lying below 20°. The variation in the $\theta$ values might also indicate that there occurs some differences between the surface properties of the various cells, though the difference is small in magnitude.

The observation that there occurs some differences in the values of $\gamma_{cv}$ when the SF9 cells were grown in the presence of Pluronic F68 (both in serum-containing medium and in serum-free medium), indicates there might be some interaction between the Pluronic F68 molecules with the surface of the cells. There are also evidences in the
literature that there occurs some interaction between the additives in a culture medium
and the cells grown in it. Considering these results, experiments were conducted to study
the lipid profile of whole cell extracts by chromatographic techniques. Thin-layer
chromatography was used for our experiments. The membranes of the cells are
composed of amphipathic molecules, such as lipids and proteins, and the nature of the
protective additives is also amphipathic in nature. Therefore, there is a possibility of
interaction between these two groups of molecules. Moreover, any change in the lipid
profile of the cell membrane will change the physical properties of the membrane, and
this will definitely change the interfacial tensions. Preliminary studies were not
conclusive. Experiments conducted with a single solvent system (chloroform-methanol-
water) did not reveal any significant differences in the lipid profile. Further examinations
need to be done with different solvent systems to investigate the possibilities of changes
in the lipid composition.

6.1 Prediction Of Cell-Gas Adhesion

From the experimental data available for a particular cell line, it is possible to
predict whether a particular protective agent will be effective in preventing cell damage.
The procedure indicated below gives an outline that can be followed:

a) Determine the values of $\gamma_{lv}$ of the liquid medium without protective additives and
the contact angle ($\theta$) that the liquid makes on a monolayer of cells;
b) Use a suitable empirical equation to calculate $\gamma_{cv}$ and $\gamma_{cl}$;
c) Use the thermodynamic relationship to calculate $\Delta F^{\text{adh}}$ to check whether the cells
would adhere to the air-medium interface;
d) If the value of $\Delta F^{\text{adh}}$ obtained is negative, protective additives, like Pluronic F68,
can be added;
e) Measure the $\gamma_{tv}$ for the same liquid medium with protective additive at a specific concentration;

f) Using the value of $\gamma_{cv}$ determined previously from step (b) to calculate $\gamma_{cl}$ using one of the empirical equations;

g) Repeat step (c) to check $\Delta F^{adh}$. If $\Delta F^{adh}$ is still negative, increase concentration and/or change protective additive.
REFERENCES


Chattopadhyay, D., Rathman, J., Chalmers, J.J. (1995a). The protective effect of specific medium additives with respect to bubble rupture. Accepted for publication in *Biotech.*


