UNIVERSITY
OF MANITOBA

Faculty of Graduate Studies
Department of Microbiology

UNIVERSITY
OF MANITOBA

The Effect of the non ionic Surfactant Pluronic F68 on Mammalian Cells in Culture

By

Hazem Ghebeh

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirement for the degree of

Doctor of Philosophy.

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

THE EFFECT OF THE NON IONIC SURFACTANT PLURONIC F68 ON MAMMALIAN CELLS IN CULTURE

BY

HAZEM GHEBEH

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

Doctor of Philosophy

Abstract:

Pluronic F68 is a synthetic non-ionic surfactant that has been used since the sixties to protect cells in culture from damaging hydrodynamic forces. It has been shown that it can protect cells by several mechanisms. These mechanisms of action have been studied mainly in hybridoma and insect cells with fewer studies made on CHO cells and other cell lines. The roles of different mechanisms of action with different cell lines and different Pluronic F68 concentrations are rarely studied and therefore are the subject of this thesis.

By developing a simple but effective assay, the effect of Pluronic F68 on the cell hydrophobicity index of CHO and CC9C10 hybridoma cells were measured. The results suggest that Pluronic F68 decreases cell to bubble interactions at least partially by decreasing the cell hydrophobicity index, which reflects a decrease in the cell surface hydrophobicity. This effect was different with the tested cell lines suggesting a direct interaction of the cell membrane with the surfactant.

In a viscometer that can produce high shear rates in the absence of bubbles Pluronic F68 increased the cells resistance to shear indicating a direct strengthening effect on the cell membrane. The effect was dose dependent at least in one of the cell lines with a full protection at 0.1% concentration.

The molecular interactions of the cell membrane with the polymer were further investigated. The work here shows that most of the Pluronic molecules are dissolved in the medium with only a very small fraction interacting with the cell membrane (if any). However, this small fraction had a significant effect on the cells hydrophobicity, nutrient uptake of the cells and the strength of the cell membrane.

The effects of other cell protectants were also studied in this thesis and were compared with Pluronic F68. Results show that while BSA has an effect on decreasing cell surface hydrophobicity like Pluronic F68, it has no effect on increasing the cell membrane resistance to hydrodynamic forces. Linoleic acid, an additive that also protects cells was not as effective as Pluronic and its effect was not significant enough when tested at very high shear forces in the absence of bubbles.

Dedication.

This work is dedicated to the Muslim Community of Winnipeg who made my stay in this city enjoyable, to my parents for their endless support, to my sisters Roula and Arwa for their friendship, and to my wife Ekhlass for everything.

Acknowledgments:

The first to thank while writing this thesis is God (Allah) for helping me achieve my goals and guide me in this life, then to my supervisor Professor Michael Butler for his patient, care, continuous encouragement, motivation, respect for his students, and extraordinarily human nature. I appreciate the help and supervision of my advisors Dr. Betty Worobec and Dr. Phil Hultin. In Prof. Butler's lab I would like to thank Norm Huzel for his excellent technical services and friendly nature, and my colleagues Ming Yang, Trish Magsino, Ming Dong Wang, Adam Burgener and Veronica Restelli for making the lab joyful and sharing their precious thoughts. Finally I want to acknowledge the help of Dr. Brian Hasinoff in the Faculty of Pharmacy for lending me his expertise in running the BRUKER instrument and Dr. Dennis Murphy in the Department of Statistics for performing the ANOVA using SAS for some of the data.

Table of Contents

List of Figures

List of Tables

Abbreviations:

AMP	Adenine MonoPhosphate
ATCC	American Type Culture Collection of Cells
BATH	Bacterial Adhesion to Hydrocarbon
BSA	Bovine serum Albumin
BT	Butyrate
C	Celsius
C.V.	Coefficient of Varience
CHO	Chinese Hamster Ovary
CHO 81	Chinese Hamster Ovary cells clone EPO-2-5-81
CHO 84*	Chinese Hamster Ovary cells clone EPO –2-4-84*
CNJsfm 2.1	Cangene serum free medium version 2.1
$CoCl_2$	Cobalt Chloride
CSH	Cell Surface Hydrophobicity
DHFR	DihydroFolateReductase Enzyme
DMEM	Dulbecco's Modified Eagles Medium.
DOT	Dissolved Oxygen Tension
DNA	Deoxy ribonucleic acid
EPO	Erythropoietin
ESR	Electron Spin Resonance (= EPR)
FBS	Fetal Bovine Serum
Fig	Figure
GMP	Guanine MonoPhosphate
HCl	Hydrochloric Acid
HGPT	Hypoxanthine-guanine phosphatidyl transferase
HLB	hydrophilic lypophilic balance
HT	Hypoxanthine and Thymidine
IMP	Inosine MonoPhosphate
IU	International Units
L	Liter
LA	Linoleic Acid
Mab	Monoclonal antibody
MilliQ	Distilled water with ions < 0.2 ppm
min	minute(s)
mL	milliliter

μL	microliter
M	Molar
mM	milli molar
μM	micro molar
n	number of trials
nL	nano liter (10^{-9} Liter)
nM	nano molar
nmole	nano mole
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.05% Tween
pmole	pico mole (10^{-12} mole)
PMF	Plasma Membrane Fluidity
rpm	round per minute
RPMI	Rose Park Memorial Institute (medium)
SD	Standard Deviation
SEM	Standard Error of the Mean
sec	second(s)

Å	Angstrom
c	side length in a triangle
Ci	Curi, a unit of radioactivity
di	impeller diameter
p	side length in a triangle
Re	Reynold number
β	Shear rate
γ	Viscosity
δ	Angle
θ	Angle
μ	micro
π	pi
τ	Shear stress

Chapter 1 : Literature Survey

Section One: Animal Cells in Culture and Shear Forces

1. Animal cell culture

Animal cells are cultured for many reasons in the laboratory. Cells growing *in vitro* can provide a model that is comparable to normal living cells in human or animal body in general (*in vivo*). In industry, the culture of animal cells is an important tool to produce recombinant proteins and other biologicals for therapeutic purposes, as diagnostic agents or simply as reagents in the laboratory.

Animal cells in general have the advantage over bacterial or yeast cells of being able to achieve posttranslational modifications of proteins or polypeptides. One important example of posttranslational modification is glycosylation of proteins to form glycoproteins. However animal cells lack a strong cell wall making them more fragile, their growth is slower and their nutrient requirements are more complex. Because of their slow growth, cultures are more prone to be over taken by microbial or fungal contaminants (Butler 1990).

Among animal cells, mammalian cells are particularly desirable for producing products for therapeutic uses. This is because of the similarities in the synthetic processes between human and other mammalian cells.

1.1 Types of animal cells in culture

Cultured cells can be divided into two general types: primary and continuous cultures. Primary cultures are those recently isolated from tissue or organ and usually have a limited lifespan in culture. Continuous cultures (cell lines) are those, which are either derived from a tumor or have been manipulated to acquire an infinite life span. Cell banks like the American Type Culture Collection (ATCC) have over 4000 cell lines.

Cultured cells can also be divided according to the method of culture into adherent (anchorage-dependent) or non-adherent cells (suspension cells). Adherent cells attach to the plastic substratum of a flask, plate, or microcarrier beads and therefore need to be detached from this surface before they can be sub-cultured. Suspension cells, on the other hand, do not normally attach to the surface of the culture vessel and can be subcultured directly by diluting with fresh medium (Morgan et a1. 1993). Examples that are relevant to this thesis are the two cell lines Chinese Hamster Ovary (CHO) cells and Hybridoma CC9C10 cells.

Chinese Hamster Ovary cells were isolated in 1957 by Puck et al. (1958) from a biopsy of an adult Chinese hamster ovary but the growing cells were more of a fibroblast-like morphology. Ham's F12 growth medium was used to clone the cells in the presence of fetal bovine serum (FBS) ranging from 4-8% (Gottesman 1985). The CHO cells have a requirement for proline (proline auxotroph) and have a modal chromosome number of 20 (ATCC catalogue).

CHO cells are mammalian non-tumor derived cells; they grow as attached or in suspension depending on the growth medium, culturing substratum and agitation speed. They have been used extensively for recombinant protein production because they achieve glycosylation in a manner similar to human cells and have been licensed as an approved cell line for synthesis of therapeutic products. However CHO cells have a tendency to clump especially in sub-optimal growth conditions (Renner et al. 1993) and they are fastidious in their growth requirement making it difficult to develop a serum free formulation. CHO cells grow best at 37° C but can still grow fairly well in temperatures between 34 and up to 39 °C. The optimum pH for growth is between 7.5 and 7.9 (Gottesman 1985).

CC9C10 hybridoma cells are mouse/mouse hybridomas derived from SP/2 myeloma cells. The hybridoma cells produce antibodies against bovine insulin. Cells grow in suspension and they produce IgG1 with a kappa light chain (ATCC catalogue).

1.2 The growth environment.

In order to culture cells *in vitro*, the environment needs to be as close as possible to that expected *in vivo*. Important environmental factors are the substratum upon which cells grow, the medium in which cells are surrounded and the temperature. There are different culture systems that can be used. The most common are stirred tank and air-lift reactors. The term "Bioreactor" is used to describe the vessels where cells are cultured in high density while certain pH, dissolved oxygen and temperature is maintained (Gor 1993, Morgan et al. 1993) for optimal growth and highest product yield.

There are different types of media from which one can choose. Most of them basically contain:

- Inorganic salts and trace elements to adjust the osmolarity and provide elements important in metabolism.
- Amino acids for protein synthesis (certain amino acids like glutamine can be used also for energy).
- Carbohydrates especially glucose to provide energy for the cell.
- Lipids and derivatives to build a functional and robust cell membrane.
- Vitamins as cofactors for metabolic pathways.
- Buffers to control and an indicator to monitor the pH (Butler 1990).

Animal blood serum is added to cell culture media up to 10% v/v (occasionally up to 15% or higher). Serum provides nutrients, protects cells from mechanical damage (shear forces) and most importantly stimulates cell growth and promotes attachment (for anchorage-dependent cells). However, serum is chemically undefined, variable between batches, and expensive, especially fetal bovine serum. Furthermore serum makes the extraction of extra-cellular released proteins more difficult and might contain some infectious particles like prions that are hard to screen for. Serum can be substituted by other additives, examples are hydrocortisone, insulin, transferrin, ethanolamine, and selenite (Butler 1990).

1.2.1 Approaches to substitute Serum:

The problem of substituting serum is knowing exactly which of the serum components are important for the growth of cells. This can be different for different cell lines and even for different clones of the same transfected cell line. There is no standard method for developing a serum-free medium; however there are two approaches. One way is to find a medium for related cells and then modify it until a suitable recipe is reached. The other method is to try one or a combination of a basal medium like DMEM, F12 or RPMI and then fortify with serum substitutes like insulin, transferrin, selenium, prostaglandins..etc. A factorial design can be used to try many additives simultaneously to save time and detect any interactions between the different additives (Freshney 1994).

2. Effect of shear forces on animal cells

The word shear by itself is sometimes ambiguous. Shear forces (τ) are defined as the forces that tend to cause sliding of adjacent layers relative to each other, in a material (Figure 1.1, Cherry et al. 1986).

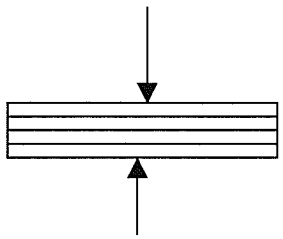


Figure 1.1 Shear forces: forces causing sliding of adjacent layers relative to each other in a material.

Shear rate (γ) is the shear stress that is caused by a velocity gradient in a direction perpendicular to the direction of flow. Where β is the liquid viscosity, the relation between shear stress and shear rate could be defined by the equation (Cherry et al. 1986):

$$\tau = \beta \gamma$$

The sensitivity of cells to shear stress depends on the cell type, the method of culture, culture medium, and culturing vessel. An example is murine hybridoma, which can sustain a constant shear stress of 0.33 N/m^2 for 14 hours without any apparent effect (Smith et al. 1987). On the other hand, insect cells are generally more prone to damage by shear stress than mammalian cells and cell lysis can happen at low shear stress of 0.1 N/m^2 (Goldblum et al. 1990). Mammalian cells in some culturing vessels (bioreactors) might experience shear stresses in bioreactors of up to 2 N/m^2 (Frangos et al. 1998).

The shear stresses experienced by the cell membrane depend also on the type of flow in a stirred cell culture, which can be turbulent or laminar. Turbulent flow is an irregular flow and occurs at a relatively high velocity while laminar flow is an orderly streamlined flow and occurs at low velocity. The Reynold numerical value determines whether the flow of stirred culture medium is laminar or turbulent. Re> 1000 denotes a predominantly turbulent flow (Papoutsakis et al. 1991). Reynold number (Re) depends on the velocity, viscosity, and the bioreactor geometry of stirred cell culture system.

$$Re = ndi^2/\beta$$

Where n is the agitation speed (in rev/s), di is the impeller diameter and β is the viscosity.

In fluid with a turbulent flow the transfer of kinetic energy from one part to the other is made through eddies (swirling pockets of fluid). With higher velocities, the eddies become smaller and smaller and there is a critical size, called kolmogorov eddy size (η), below which mechanical energy dissipates into heat. (Papoutsakis et al. 1991).

2.1 Effect of shear on suspended cells:

2.1.1 In the presence of air-liquid interface:

In mammalian cell biotechnology, cost effective production of biologicals is achieved by large-scale high-density cultures. In these large-scale cultures the demand for oxygen is high and surface aeration is usually not sufficient to transfer oxygen through the air-liquid interface especially for bioreactors larger than 2 Liters. Bubble sparging is the easiest and the most effective method of increasing dissolved oxygen tension (DOT) in the medium (Zhang et al. 1992). However, sparging generates bubbles that burst at the air liquid interface at the top surface (death zone) leading to detrimental effects on cells (Kunas et al. 1990, Trinh et al. 1994, Jordan 1994).

Several mechanisms have been proposed for the damaging effect of bubble breakup. They include (1) shearing of cells in the thin draining liquid films of the foam (Handa-Corrigan et al. 1989; Cherry et al. 1992), (2) the fast retracting rim during the bubble rupture (Cherry et al. 1992), (3) and the subsequent formed liquid jet (Dey et al. 1997). The draining liquid films (lamellae) reduce to a thickness less than the diameter of the cell leading to cell deformation or probably flattening of the cell. Once the lamellae rupture, the retracting rim rapidly accelerates cells from stationary state to high velocity calculated to be 3 m/s. This

acceleration produces hydrodynamic forces that have sufficient magnitude to cause cell death. The fluid flow from liquid jet is estimated to produce a force of 200 N/m^2, which is also sufficient to cause cell death (Cherry et al. 1992). Trinh et al. (1994) has calculated the number of killed cells to be 1050 cells per single 3.5 mm bubble rupture when culturing SF-9 insect cell in TNM-FH medium (Figure 1.2).

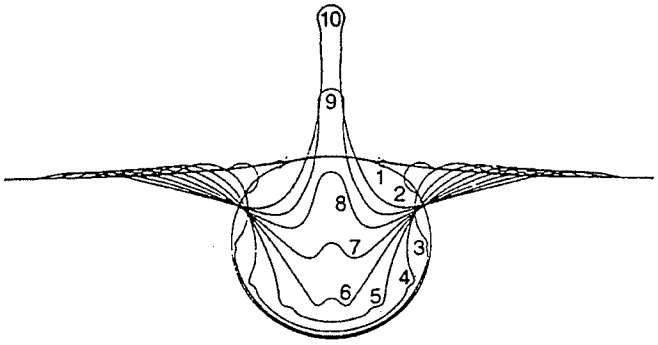


Figure 1.2 Time sequence of breakup of a 1.7 mm bubble in sea water. Bubble-surface profiles are approximately 1/6000 s apart. MacIntyre. F. 1968

The evidence for the damaging effect of bubble breakup at the top surface has been reported by many including Handa-Corrigan et al. (1989), Oh et al. (1992), Kunas et al. (1990) and Michaels et al. (1996). Handa-Corrigan et al. (1989) have shown that bubbles have less detrimental effect on cells in longer bubble columns than in shorter ones. This is due to the low air-liquid interface area/volume ratio. Kunas et al. (1990) and Michaels et al. (1996) have demonstrated that in a completely filled bioreactor with no air-liquid interface bulk liquid bubbles have a negligible effect on cells, and therefore only bubbles bursting at the surface are detrimental to cells.

Stirring (agitation) is important to keep free cells or microcarrier beads in suspension and assure homogenous conditions around the cells as well as enhance the gas dissolution from the gas phase to the liquid phase (Cherry et al. 1986). Stirring also generates shear forces nevertheless with speeds below 600 to 800 rpm the shear forces generated from cell mixing is rarely a problem in a bubble free environment where the air-liquid interface is eliminated (Kunas et al. 1990). Otherwise in the presence of the air liquid interface agitation speeds of above 250 rpm can be a problem since a vortexing effect might be generated leading to air entrainment and therefore bubble bursting at the air-liquid surface.

2.1.2 In the absence of air-liquid interface:

With certain bubble free aeration devices, the air-liquid interface can be eliminated. The oxygen can be delivered through membrane tubing, which permits the diffusion of oxygen from the gas phase to the liquid phase. In such systems the culture could be agitated up to 600 rpm without extensive cell death although some of the bubbles might be formed in the bulk of the medium (Michaels et al. 1996).

Above 800 rpm and in the absence of air-liquid interface the main mechanical forces acting on the cells are due to agitation. At such speeds the komolgorov eddies formed from the impeller are smaller than the cells (Papoutsakis 1991 and Michaels et al. 1996) and energy dissipation from these small eddies leads to cell damage (Papoutsakis 1991). Bubble-cell interaction in the bulk of the fluid has negligible effect on cell damage (Michaels et al. 1996).

2.2. Effect of shear on adherent cells:

When grown as a monolayer in T-flasks or slides, anchorage dependent cells can withstand high shear stresses (up to 20 N/m^2) if exposure time is short (<1 hour). For longer exposure times, adherent cells become sensitive even to lower shear stress levels (0.75-1 N/m^2). The responses to shear forces appear as changes in morphology, changes in the alignment of cells, shortening, piling, detachment and death. Different cell lines show different sensitivities (Kretzmer et al. 1991 and Ludwig et al. 1992).

2.2.1 Adherent cells on microcarriers:

Adherent cells can be cultured on glass or polymer beads, which is particularly useful for large-scale cultures. The beads offer a large surface area for cell attachment and if stirred provide a homogenous environment around the cells. Because of the relatively larger size of beads compared to free cells, cells attached to microcarriers are more sensitive to agitation compared to cells in suspension (Papoutsakis 1991). For instance, it was found experimentally that the maximum non-damaging stirring speed of 60 rpm in microcarrier system correlates to 250 rpm with free cell suspension culture. At 100 rpm there is an apparent growth inhibition and at 150 rpm there is severe damage to cells in microcarrier cultures (O'Connor et al. 1992).

In microcarrier systems, experimental evidence indicates that bead-to-bead interactions are the main source of cell damage in bioreactors under intense agitation conditions (Papoutsakis 1991) and in high bead concentration (Fig 1.3, Croughan et al. 1988). The next source of damage, prevalent at low bead concentrations, is "Bead-to-fluid"

interactions. It involves the collision of beads with kolmogorov eddies that are equal to or smaller than the bead size (Papoutsakis 1991 and Croughan et al. 1988). Upon collision, eddies generate energy that dissipates on the surface of the bead leading to cell damage.

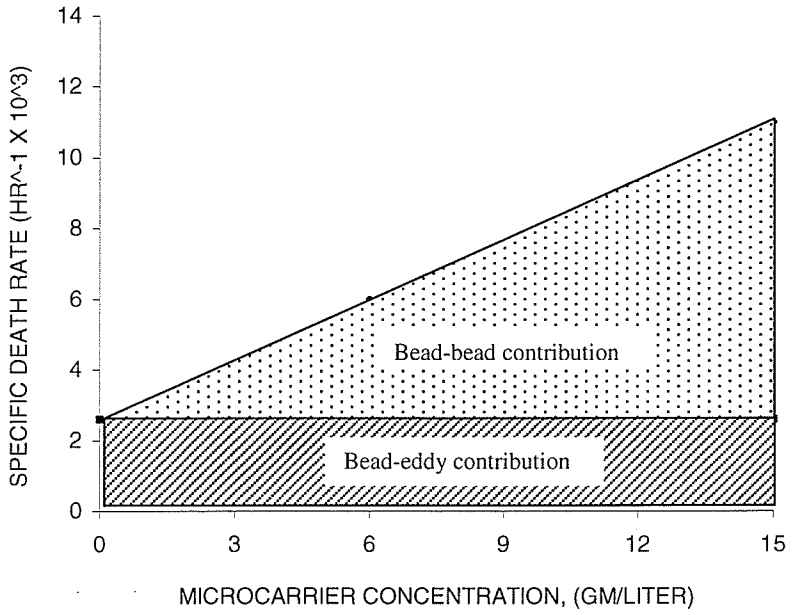


Figure 1.3 Source of damage in microcarrier system with different microcarrier concentration. Croughan et al. 1988. FS-4 cells were cultured on cytodex 1 microcarriers in spinner flasks at 35 rpm.

Fluid-to-cell interactions are of lesser importance in the damage of freely suspended cells. Interaction of beads with bioreactor elements (impeller and probes) is relatively infrequent and with a well-designed impeller, collisions between beads and the impeller do not appear to cause any substantial cell damage (Papoutsakis 1991).

3. Effect of shear on the metabolism of cells and protein expression:

In biotechnology, cell damage is frequently assessed in terms of cell death and/or reduced viability. However, it has recently been demonstrated that sublytic (non-lethal)

hydrodynamic stress that does not cause loss of cell viability in a bioreactor can affect the shape, physiology, cytoskeleton, membrane structure, DNA and protein synthesis and/or the metabolism of a number of cells (Papoutsakis 1991). For instance, HL60 (human promyelocytic leukemia) cells agitated at 400 rpm showed a decrease in both CD13 and CD33 surface proteins. The decrease might be due to physical damage of the protein, decreased protein synthesis or damaged traffic channels between the site of synthesis and the cell membrane (Lakhotia et al. 1993). Furthermore, it was shown that the secretion of htG antibody from hybridoma cells increases with higher agitation rates and it is possible that this is due to leaky cell membranes induced by sublytic (non-lethal) shear forces (Shiragami et al. 1997).

The response to sublytic (non-lethal) shear forces could be different depending on the intensity of such forces and the specific cell line. For example, in CHO cells DNA synthesis increased when the agitation speed increased from 80 to 250 rpm (Al-Rubeai 1995 et al.) while, in hybridoma cells DNA synthesis decreased with an agitation speed of 600 rpm (Al-Rubeai et al. 1990). The CHO cells agitated at 250 rpm showed also a changed distribution of cell cycle phases with an increase in S phase (DNA synthesis phase) cells and a decrease in G1 phase cells (Lakhotia et al. 1992). On the other hand, hybridoma cells agitated at 1500 rpm, showed a sharp decrease in S-phase cells and a complete loss of G2 cells (Al-Rubeai et al. 1995).

Sublytic shear forces were found to increase the metabolism of cells and their production rate of metabolites. Prostacyclin production was increased proportionally when human

umbilical vein endothelial cells were subjected to steady state stress of 0.6 to 2.4 N/m^2 (Frangos et al. 1988). On the other hand altered metabolism of the cell could increase the cells' susceptibility to shear forces. For example, altered metabolism due to accumulation of ammonia, decrease in pH, and nutrient depletion increases the sensitivity of cell to shear forces. This effect of metabolism occurs especially in stationary phases possibly by decreasing cell-repairing ability (Papoutsakis 1991 and Zhang et al. 1992). Lower temperature has a similar effect to the above mentioned changes in medium. This is likely due to lowered metabolism and slower repair mechanisms (Al-Rubeai et al. 1990).

The growth phase of the cell affects the susceptibility to shear forces. The mean bursting membrane tension, which is the tension of the cell membrane during bursting of a single cell applied between two optical fibers with flat ends, was found to increase from lag phase through the exponential phase and to decline in the death phase. (Zhang et al. 1992[c]).

4. Protection from shear forces:

A number of additives have been used to prevent or minimize the damaging effect of shear forces on cells. They include serum and serum proteins like albumin, derivatized celluloses like methylcellulose, derivatized starches like hydroxyethyl starch, dextran, Pluronics like Pluronic F-68, polyvinyl Alcohol (PVA), polyvinyl-pyrrolidone (PVP), and polyethylene glycol (Michaels et al. 1991, Michaels et al. 1995, Chisti 1999). Another candidate method that was developed in our laboratory is the addition of fatty acids to increase the membrane resistance to shear forces (Butler et al. 1999). The

protective effect of the additive depends on the properties of the specific additives, the concentration used, the type of cell in culture, and the mechanism(s) of the protecting additive (Chisti 1999). Alternatively, the head space of bioreactor can be removed, to eliminate the air-liquid interface, and coupled with an bubble-free aeration device.

The following two sections will focus on the protective mechanisms of media additives such as Pluronic F68, serum and serum derived protectants like serum albumin, linoleic acid and cholesterol.

Section two: A Cell Protectant: Pluronic F68

Pluronic is a block copolymer and is considered as a synthetic nonionic surfactant. As a copolymer it has two different monomers, ethylene oxide and propylene oxide. It is also a block copolymer because of the arrangement of the monomers. Pluronic has a central hydrophobic polypropylene oxide group sandwiched between two lateral, hydrophilic polyethylene oxide groups (Fig 1.4). There are different types of Pluronic designated with the letter F, L, or P to indicate the physical state of the polymer (F for flake, L for liquid, and P for paste) followed by a number of two or three digits. The first one or two digit(s) represent the molecular weight of the hydrophobe with numbers 6, 8, and 12 denoting molecular weights of 1750, 2250, and 4000 respectively. The last digit represents the percentage of the hydrophile divided by 10. Pluronic F-68 (also known as poloxamer-188) has a hydrophobe (polypropylene oxide) molecular weight of 1750 and hence the first digit of 6. The molecular weight of Pluronic F68 is 8400, of which polyethylene oxide forms 80% of it and hence the last digit is 8 (BASF Publication 1996; Murhammer and Goochee 1990[a]; Krevelen 1972).

$$OH\ (CH_2\ CH_2\ O)_a\ (CH\ CH_2\ O)_b\ (CH_2\ CH_2\ O)_c\ H$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\quad CH_3$$

Figure 1.4 General chemical structure of Pluronic. For Pluronic F-68 average values of a, b, and c are: 75, 30 and 75 respectively (BASF 1996).

1.0 The Protective mechanism of Pluronic F68

The protective role of pluronic-F68 has been attributed to either one or a combination of the following phenomena: (a) a reduction in the cell-to-bubble attachment (Michaels et al. 1995); (b) a reduction in the fluid-mechanical forces encountered by cells near the rupturing bubble (Dey et al. 1997); (c) a stabilizing effect on the surface foams to prevent the damaging bubble events (Handa-Corrigan et al. 1989); and (d) an increased cell resistance to shear damage (Murhammer and Goochee 1990[b]; Zhang et al. 1992[b]). The main mechanism in a sparged stirred tank reactor was found to be the reduced cell-to-bubble attachment (Michaels et al. 1995). The second above mentioned mechanism is purely physical in nature and it depends on reducing the surface tension of the liquid. The "stabilizing foam effect" might be applied only when there is a stable foam on the air-liquid interface which usually does not happen with mammalian cell cultures due to lower rates of oxygen consumption and therefore rates of sparging. Due to their importance and relevance to this thesis the first and last mechanisms will be discussed below in more details.

1.1 Decreasing cell-to-bubble attachment:

Kunas and Papoutsakis (1990) have shown that the major shear producing effect on cells in a culture system comes from bubble breakup at the air-liquid interface near the top head space of the bioreactors. They have shown clearly that when the headspace of the bioreactors is eliminated the culture can be agitated up to 600 to 800 rpm without significant effect on the cell growth. On the other hand, in the presence of headspace cells are destroyed in the air-liquid interface when the culture is bubble sparged or if the

culture is agitated above 200 to 250 rpm due to vortexing and therefore introduction of bubbles into the medium.

It has been reported that the most important mechanism of Pluronic F68 in protecting mammalian cells from the detrimental effect of bubble sparging is decreasing the cell-to-bubble attachment. Michaels et al. (1995) have found that Pluronic F68 decreased the cell to bubble attachment as measured by three different parameters:

i) An increase in cell induction time (time needed for 50% of cells in culture to attach to bubbles),

ii) Decrease in cell entrapment in the thin film of bubble lamellae (or a quick escape from the draining film lamellae)

iii) Decrease in cell-carry over with bubbles as determined by a foam flotation assay. In this experiment, foam generated from bubbling in one container was transferred to another container and let to settle. The number of cells in the second container (C_{foam}) over the original cell number (C_{bulk}) in the first container determined the foam ability to carry cells (a_{sep}).

$$a_{sep} = C_{foam} / C_{bulk}$$

Jordan et al. (1994) found that saturation of bubbles with a surfactant like Pluronic F68 depends on the concentration of the surfactant in the medium and the time the bubble stays in contact with the medium. They found that:

- Fully saturated bubbles resulted in no cell to bubble contact.
- Partially saturated bubbles contacted the cells but did not damage them.

- Unsaturated bubbles with surfactants were always lethal to CHO cells upon contact

Cells attached to bubbles can migrate with the bubble to the top of the reactor where they might be destroyed upon breaking up in the air-liquid interface.

Michaels et al. (1996) in a later work tried to examine the effect of bubble coalescence in the bulk medium where the head space of the reactor is eliminated and therefore the effect of bubble bursting in the Air-Liquid interface is avoided. This group found that although some cell death might occur in the bulk medium this was minor and it had no significant effect on the growth and viability in the medium. This happened in a serum fortified and serum free medium and at low agitation rates of 100 rpm and higher agitation rates of 400 rpm.

Meier et al. 1999 provided a model where the contact of cells to rising bubbles has an important effect on cell death. Cells attached to rising bubbles are destroyed upon bubble bursting. This was experimentally verified by trying bubble columns with different heights but the same volume. With the same amount of bubbles generated the death rate was higher with higher columns. Pluronic F68 when added decreased the cells' attachment to rising bubbles and made the bubble killing effect independent of column height. Although this was contrary to what has been found by others (Handa Corrigan et al. 1989; Tramper et al. 1988), Meier et al. argues that this effect is more evident with longer reactors and in the absence of any protective additives. Handa-Corrigan et al.

(1989) used columns with height/volume ratio of 30 while Meier et al. (1999) used columns with height/volume ratio of 111.

1.2 Increase in the cells' resistance to shear forces

Zhang et al. (1992) have shown using micromanipulation techniques that the tension required to burst hybridoma cells increased significantly in the presence of Pluronic F68. Goldblum et al. (1990) reported the same effect on insect cells using a modified rheogoniometer (a device with a rotating plate and a stationary cone) using higher concentrations of 0.2 and 0.3%. On the other hand Michaels et al. (1991) reported no effect of 0.1% Pluronic F68 with hybridoma cells measured by a viscometer.

The increased resistance was suggested to be by a rapid adsorption of the Pluronic on the cell membrane as well as by a slow incorporation of Pluronic in the cell membrane (Zhang et al. 1992[b]). The rapid adsorption of Pluronic could also plug already damaged parts of the cell membranes as was suggested by Al-Rubaei et al. (1993) based on the observation that Pluronic reduced the uptake of fluorescein dye by damaged cells in highly agitated cultures.

2.0 Effect of Pluronic F68 on cell growth and recombinant protein production:

In vitro Pluronic F68 inhibited DNA synthesis and hence growth in TB/C3 and PQXB1/2 hybridoma cells as shown by the uptake of thymidine into DNA. These observations explained the reduced growth by 12% in continuous culture spinner flasks in the absence

of bubbles of these hybridoma cells at 0.05% Pluronic F68 concentration (Al-Rubeai et al. 1992). On the other hand, Keen et al. (1995) experienced a growth stimulatory effect of Pluronic F68 on CHO cells cultured in T-flasks. Bentley et al. (1989) also reported a stimulatory effect for both melanoma and Chick Embryo Fibroblasts (CEF) in stationary cultures.

Several researchers tried to explore this effect of Pluronic F68 on cell growth. Bentley et al. (1989) noted the presence of impurities in commercially available Pluronic and they further purified it on a silica gel column. The unpurified (commercial) Pluronic F68 was mildly stimulatory for CEF and melanoma cells as noted above. After purification Pluronic F68 became inhibitory for CEF cells and very stimulatory for melanoma cells. Some of the low molecular weight impurities were identified: aldehyde and both formic and acetic acids (Bentley et al. 1989).

Weeratunge (1993) and Handa-Corrigan and Weeratunge (1997) studied also the effect of impurities in the Pluronics and have found that both unpurified and purified Pluronic were cytotoxic to cells in culture. They further identified butylated hydroxy toluene (BHT) and phthalate as some of the impurities responsible for the inhibitory effect on cells. BHT is an oxygen radical scavenger (antioxidant) commonly used as a food preservative while phthalate esters are commonly used as plasticisers, which impart flexibility to polymers. They concluded that both purified and unpurified Pluronics were cytotoxic to cells and were responsible for alterations in cell morphology. However in their study very high concentrations of Pluronic were used, ranging from 0.2% to 1.6%.

In cell cultures much lower concentrations of 0.05% to 0.2% are used. Therefore such an inhibitory effect might not be relevant to cell culture conditions. Furthermore, the Pluronic F68 was purified by a supercritical fluid extraction (SFE) with hexane as a solvent. Hexane by itself is cytotoxic and traces of it might remain after extraction. Therefore any toxic effect from the purified Pluronic might be from the residual hexane (Weeratunge 1993; Handa-Corrigan and Weeratunge 1997).

In vivo Pluronic F68, more known as Poloxamer 188 in clinical and formulation studies, has been used as a food additive (Hoseney et al. 1972), stool softener (ex. Codalax®; Douglas pharmaceuticals Ltd.), topical wound cleanser (ex. Band-Aid®;), as an emulsifying agent in IV fat emulsions, and as an excipient in many pharmaceutical formulations (ex. atovaquone and irbesartan) and vaccines (Moghimi et al. 2000). Poloxamer 188 also has a hemorheological and antithrombotic properties that reduce (as an adjuvant) myocardial infarction in animal models. Poloxamer 188 (RheothRx by Glaxo Wellcome; 150 mg/mL intended for IV administration) was shown to be safe in a pilot study to help in acute painful episodes of sickle cell discease. When given orally or IV it is excreted (unchanged) mainly in the urine. Fluosol®, which contains 2.7% Pluronic as an emulsifying agent, is approved now for the prevention or diminution of myocardial ischemia during coronary angioplasty in patients at high risk of ischemic complications (Jewell et al. 1997).

Anaphylactoid reactions have been reported after Fluosol infusion due to activation of complement. This effect was also found after a bolus injection of Pluronic F-68 and an

inert dense liquid (mineral oil). It is believed that Pluronic creates a certain configuration around dense liquid micelles, which triggers the activation of complement (Ingram et al. 1993; Smith II et al. 1987). Paralysis of phagocyte migration is another side effect of Fluosol® and it was found that it is entirely due to Pluronic F-68. The ID_{50} for phagocyte migration, chemotaxis, and adhesion was 1.4, 2.4, and 2.9 mg/mL respectively (equivalent to 0.14%, 0.24%, and 0.29% of Pluronic, respectively; (Lane et al. 1984 and 1986). Some reports have suggested that these adverse effects might be due to some impurities in the Pluronic F68 preparations. However, until now these effects are not proven.

The above shows that Pluronic F68 has an effect on the growth of some cell lines that is independent of its protecting effect in sparged cultures. *In vitro,* the effect on growth is dependent on the cell line, the method of culture, Pluronic F68 concentration, and possibly the purity. On the other hand, the effect of Pluronic F68 *in vivo* might be more variable because of the variability of living body. Factors like concentration, route of administration, and possibly purity, might be crucial. Caution should be taken not to mix Pluronic F68 with other Pluronics since the effect of these compounds on cells (either *in vivo* or *in vitro*) might be completely different (Muhammer and Goochee 1992[a]; Kier et al. 1995). Furthermore, the effect of Pluronics on cells *in vivo* does not necessarily reflect their effect on cells in vitro and vice versa (Kier et al. 1995).

Section Three: Other cell protecting agents

Serum has been shown by many researchers to provide protection from shear forces in bubble-sparged cultures for both CHO and hybridoma cells (Martens et al. 1992, L. Van der Pol et al. 1992, McQueen et al. 1989, Michaels et al. 1991). The protection has been found to be mainly "physical" although some "biological" effect was also detected. The "physical" effect is expected to occur very soon upon serum addition because it is the result of interactions with the molecules in the liquid medium, the bubbles and possibly the cells. On the other hand the "biological" effect is expected to take a relatively longer time because it is dependent on a slower process in the cell membrane and/or cytoskeleton of the cells to make it more shear resistant.

The rapid protective effect of serum addition is shown to be by a decrease in the cell-to-bubble attachment (Jordan et al. 1994). This was found by using 5% serum to saturate the bubble air-liquid surface with the serum components. Furthermore Handa Corrigan et al. (1989) suggested "the stable foam mechanism" where serum or other cell protective additives can stabilize the foam at the upper surface of the bioreactor forming a stable foam shelter to prevent the damaging effect of bubble breakup on the cells. However that only applies in the presence of a stable foam layer, which does not always occur in mammalian cell culture. Such a foam layer is more likely to develop in bacterial fermentation cultures where sparging rates are much higher or in high percentage serum supplemented media due to high protein content.

On the other hand, in the absence of bubbles and using a viscometer, a device that can expose the cells to high shear forces in the absence of bubbles, the immediate short term "physical" effects were not detected. Only the long-term "physical" or "biological" effects were apparent (Michaels et al. 1991). This long term effect could be by a change in plasma membrane fluidity as proposed by Ramirez et al. (1990) who found a correlation between the plasma membrane fluidity and the shear sensitivity of mammalian cells. Furthermore serum contains many nutrients that could be incorporated into the cells over a long period to produce a more shear resistant and robust cell membrane or cell cytoskeleton.

Serum is composed of many nutrients, proteins, and fatty acids that can be responsible for its protective effect. Albumin, the most abundant protein in serum could be, at least partially, the active ingredient that is responsible for the serum's "physical" effect. In a recent study (Butler et al. 1999) bovine serum albumin (BSA) has been shown to protect agitated cultures of CC9C10 hybridoma cells stirred at 470 rpm. At this speed a vortex occurs in the liquid leading to bubble entrainment and a subsequent decrease in the viable cell count within a short period of 4 to 5 hours.

Serum contains many free fatty acids that can be taken up by the cell and therefore can account for any long term biological effects. Mammalian membranes are mainly composed of three kinds of lipids: phospholipids, glycolipids and cholesterol. Oleate (monounsaturated) and palmitate (saturated) form the majority of the fatty acids content of the cell membrane (Rintoul et al. 1978). However, mammalian cells have the capacity

to incorporate the exogenously provided lipids with no apparent controlling mechanisms (Butler et al. 1995). Therefore, changing the medium composition of fatty acids can change the composition of fatty acids in the membrane. While some cells can synthesize their requirements of most fatty acids, cholesterol, and proteins, others are auxotrophs with dependence on the substrate to be provided in the medium (Sato et al. 1988). The lipid composition can also affect the configuration of some membrane receptors and therefore it might affect a number of cell functions (Goodwin et al. 1990).

A recent study reported the improvement of growth and antibody production of a hybridoma cultured in media supplemented with linoleic and /or oleic acids. Furthermore linoleic acid decreased the shear sensitivity of these cells in highly agitated spinner cultures (470 rpm). The decrease in the shear sensitivity is shown to be after a significant change in the fatty acids (including linoleic acid) composition of cell membrane (Butler et al. 1997; Butler et al. 1999).

There are different effects reported for lipids on mammalian cells. For free fatty acids, some reported an increase in the growth rate of the cells (Castro et al. 1995, Savonniere et al. 1996, Butler et al. 1995) while others reported an increase in the recombinant protein production (Savonniere et al. 1996, Schmid et al. 1991). The difference in the response of mammalian cells can be attributed to the difference in concentrations used (Butler et al. 1995), different clones of cells (Mackinnon et al. 1992) and to different recombinant proteins they produce. Unsaturated fatty acids like linoleic acid increase the cell membrane fluidity while saturated ones have the opposite effect. For example, it was

found that supplementing the growth medium of CHO cells with a saturated fatty acid like palmitate increased the solid phase lipid while supplementing with oleate (monounsaturated) had no effect (Rintoul et al. 1978). Furthermore, the type of added lipid can affect the cell very differently. For example, phosphatidylserine has been reported to induce apoptosis in the CHO cells while other forms of phospholipids had no effect (Uchida et al. 1998).

Section Four: The aims of the research:

1. Purpose:

Many researchers have studied the protective effects of Pluronic F68 in sparged large-scale cell culture. Most of the studies were done on hybridoma or insect cells and few on CHO cells. However, there has been no comparison made between the effect of Pluronic F68 on different cell lines and the effect of different concentrations of Pluronic F68. An optimal concentration of Pluronic F68 may be appropriate for each cell line. In this thesis attempts are made to show the effect of Pluronic F68 on a variety of mammalian cell lines at different concentrations. Furthermore each mechanism has been explored separately.

2. The Aims of the Research:

1. **To Study the effect of Pluronic F68 on CHO cells growth and recombinant protein production.**

Keen et al. (1995) have observed a growth stimulatory effect of Pluronic F68 on CHO cells cultured in T-flasks. Bentley et al. (1989) have reported a stimulatory effect for both melanoma and Chick Embryo Fibroblasts (CEF) using the commercially available Pluronic F68. Castro et al. 1996 also reported a stimulatory effect of Pluronic F68 on the growth of CHO cells only in the presence of 1 mg/mL BSA and not in the presence of 5 mg/mL BSA. On the other hand Al-Rubeai (1992) has reported an inhibitory effect for hybridoma TB/C3 and PQXB1/2 cells. These conflicting results might be due to the different cell lines used, the culture mode of these cells, different growth media, and

possibly batches of Pluronic F68. Bentley et al. (1989) used anchorage dependent cells, Castro et al. (1996) cultured CHO cells as suspension cells in agitated flasks, while hybridoma cells in Al-Rubaei et al. (1992) were suspension cultures in spinner flasks. CHO cells were chosen for our study as the cells can grow both as adherent and in suspension depending on the culturing method and the medium. The aim was to study the effect of Pluronic F68 on the same cell line under different modes of culture.

2. To study the protective mechanism of Pluronic F68 in the presence of bubbles.

Pluronic F68 has been shown to decrease the cell-to-bubble attachment, however it is still quite controversial whether this is a purely physical effect on the bubbles or if an interaction between Pluronic F68 and cell membrane also contributes to this mechanism. To study this further we have chosen two different cell lines (CHO and CC9C10 hybridoma) with different intrinsic biological cell membrane compositions and developed an assay whereby the interaction of these cells with a hydrophobic surface like the air-bubble can be measured.

3. To study the protective mechanism of Pluronic F68 in the absence of bubbles.

Pluronic may also increase cell membrane strength although this phenomenon is controversial. Zhang et al. (1992) have shown using micro-manipulation techniques that the bursting tension increased significantly in the presence of Pluronic F68. On the other hand, Michaels et al. 1991 reported no significant protective effect of Pluronic F68 in the absence of bubbles. The latter group investigated this using a viscometer that produced high shear forces in the absence of bubbles.

The objective of this study was to investigate the protective effect of Pluronic F68 in a bubble free environment. The study was performed in stationary and agitated cultures with minimum shear forces compared with agitated cultures with higher shear rates. To study the protective effect at high shear rates but in the absence of bubbles, a viscometer with high shear forces was used and was tested on two important cell lines, Chinese Hamster Ovary cells (CHO) cells and CC9C10 hybridoma cells.

4. To study the effect of Pluronic F68 on nutrient uptake.

Mizrahi 1975 suggested that in addition to its protective effect, Pluronic F68 can increase nutrient uptake into and hence growth of cultured human lymphocytes. However, this was not shown with any nutrient.

5. To study the molecular interactions of Pluronic F68 with the cell membrane.

The interaction of Pluronic F68 with the cell membrane has been supported by many reports (Murhammer and Goochee 1990; Zhang et al. 1992; Wu et al. 1997). However the details of this interaction on the molecular level are still unclear.

6. To study the protective effect of BSA, linoleic acid and compare with Pluronic F68.

BSA and linoleic acid have been reported to protect cells from shear forces. However it is not known whether this is a similar effect to Pluronic F68 or different. Recently free unsaturated fatty acids have been explored as additives to serum free cultures to replace

growth-promoting properties of lipids in serum. One of these fatty acids, linoleic acid when complexed with BSA, acting as a carrier molecule, and added in μM concentrations to serum free cultures of hybridoma cultures, increased cell yields in agitated and stationary cultures. Linoleic acid has also been shown to increase the cell robustness in high shear forces. However the mechanism is unclear.

This study will explore the growth enhancement of CC9C10 hybridoma cells in μM concentrations of cis-unsaturated linoleic acid. The subsequent effect on cell surface hydrophobicity will be assessed by performing a simple but effective hydrophobic cell-partitioning assay.

3. The direct Objectives of the Research:

a) **Optimize a cell growth medium for CHO cells.**

b) **Examine the effect of Pluronic on the growth and recombinant EPO production of CHO cells.**

c) **Study the protective effect of Pluronic in the presence of bubbles by measuring changes in hydrophobicity index of two cell lines.**

d) **Study the protective effect of Pluronic in the absence of bubbles using a viscometer for two cell lines.**

e) **Study the effect of Pluronic F68 on nutrient uptake**

f) **To study the interaction of Pluronic F68 molecules with the cell membrane by:**

   i) **Developing a method to measure Pluronic F68 concentration in cell growth medium.**

ii) **Calculating the Pluronic F68 total surface area.**

g) **To study the protective effect of Linoleic acid and BSA on cells and compare them with that of Pluronic F68.**

Chapter 2 : Methods and Materials

1. Cell culture:

1.1 Materials:

Chinese Hamster Ovary (CHO) cells were derived from a transformed non-tumorgenic hamster ovary cell, which was isolated in 1957. A subclone called CHO-K1, which require proline in serum free media, was transfected with the human erythropoietin (EPO) gene by Cangene Corporation, Winnipeg, Canada. Two clones designated 81 and 84* were provided to Prof. M. Butler at the University of Manitoba.

Hybridoma CC9C10 cells derived from a murine B-lymphocyte and an SP/2 myeloma was used in this study. This cell line secretes a monoclonal antibody (IgG) against bovine insulin. Cells were obtained from the American Type Culture Collection of Cell lines (ATCC). SP2/0 myeloma cells were also obtained from the ATCC.

T-flasks, centrifuge tubes and serological grade pipettes were obtained from Corning, Corning, New York. Spinner flasks (100 and 250 mL) were obtained from Bellco Glass Inc, NJ, USA. Medium sterilization filters were obtained form Pall Corporation Mississauga, Canada while Nalgene small syringe filters where obtained from Fisher Scientific Limited. Canada. Ex-Cyte VLE was obtained from Miles Inc. (Now called Serologicals Inc.), Ohio, USA. Trypsin Inhibitor was purchased from GibcoBRL, Grand Island, USA. All other chemicals were obtained from Sigma unless otherwise mentioned. Trypsin was dissolved in MilliQ water and then 0.2 μm filter-sterilized. Trypsin Inhibitor was dissolved in Phosphate Buffered Saline (PBS) and then 0.2 μm filter-sterilized.

1.1.2 Growth medium.

Hybridoma CC9C10 cells were grown in serum-free medium formulation (Biogro H). This medium is proprietary and was developed in Prof. M. Butler's laboratory. For medium preparation all powders were dissolved in 80% of the total volume except for insulin, which was dissolved separately in 5 mL MilliQ water with the help of diluted HCl before being added to the medium. After complete dissolution, the volume was adjusted with the addition of MilliQ water followed by filtration through 0.2 μm filters with a peristaltic pump (Watson Marlow Model 503S).

CHO cells were cultured in a proprietary medium CNJ-sfm (Cangene serum-free medium) that was prepared by GibcoBRL. The medium contains low levels of proteins (around 120 μg/mL) and Pluronic F68 (1 mg/mL) unless otherwise mentioned (Lao et al. 1996).

Hypoxanthine and thymidine stock solution was dissolved by alkalinization with a few drops of sodium hydroxide (4 M). α-Tocopherol were dissolved in absolute alcohol and then further diluted in medium. Linoleic acid was complexed by adding 1 to 50 μL of 1.0 M linoleic acid in ethanol to a 10 mL of distilled water containing 10 mg/mL of BSA. The complex was formed by mixing in a rotamixer and shaking for 1 hour at room temperature at around 300 rpm with a shaker (New Brunswick Scientific G24 Environmental Incubator Shaker). CNJ-Sfm contains 5 μM Linoleic acid unless otherwise mentioned. Water-soluble form of cholesterol (Sigma) was used. All other supplements were freely soluble in growth medium.

All stock solutions were stored in 4 °C except for phosphoethanolamine, linoleic acid, trypsin and trypsin inhibitor which were stored frozen at –20° C.

1.2 Methods:

1.2.1 Sub-culturing:

CHO cells were cultured in 25 or 75 cm^2 T-flasks for stationary cultures and 100 mL Bellco spinner flasks for spinner cultures. The inoculation density was 10^5 cells/mL and cells were subcultured every 4 or 5 days The subculturing procedure of CHO cells required trypsinization using porcine trypsin and soy trypsin inhibitor. The cells were grown anchorage dependant in stationary T-flasks and were in suspension in agitated cultures. The cells were cultured up to a relative passage number of 50 after which the cells were discarded. The relative passage number starts from the time the cells were received from Cangene Corporation.

Hybridoma cultures were cultured in 75 or 175 cm^2 T-flasks. The inoculation density was 10^5 cells/mL and cells were subcultured every 3 days. Cells were subcultured by taking a well-mixed inoculum containing the required total cells needed for inoculation and diluting with fresh pre-warmed (37° C) medium.

A batch of cells was maintained in a growth phase by daily medium changes. The cells were used for linoleic acid uptake, shear sensitivity, and hydrophobicity measurement experiments.

1.2.2 Incubation

Cells were incubated in a humidified incubator with 8 to 10% CO_2 atmosphere at 37° C. The cap of the flask was loosened to permit the exchange of gases.

1.2.3 Cell counting

Viable cells were counted by taking a homogeneous culture sample and mixing with an equal volume of 0.2% trypan blue. The cells were counted with an improved Neubauer haemocytometer under a microscope (Nikon Labophot 2) with a magnification of x100. All reported cell numbers in this thesis are an average of two counts. In each count, cells in 4 large squares were counted and the cell concentration was calculated according to the following formula:

$$Cell\ concentration\ (cells/ml) = \frac{Total\ cell\ count \times dilution\ factor\ (2) \times 10^4}{Number\ of\ squares\ (4)}$$

1.3. Statistical Analysis:

The statistical significance of the results was determined by Analysis of Variance (ANOVA) performed by SAS program, version 8.2.

2. Pluronic Assay in culture medium:

2.1 Materials:

Pluronic F-68, cobalt nitrate, and ammonium thiocyanate were obtained from Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada. Ethyl acetate was obtained from The British Drug Houses Ltd. Poole, England. All reagents were of analytical grade.

A stock solution of 0.2% (w/v) was prepared by dissolving 200 mg of Pluronic F68 in 100 mL of distilled water or Pluronic-free medium. From the stock solution, serial standard dilutions were made. The concentrations of the standards were 0.2, 0.16, 0.12, 0.1, 0.08, 0.04, 0.02, and 0.01%. The standard dilutions were made either with water or with cell culture medium. The cobalt thiocyanate reagent (dye solution) was prepared by dissolving 3 g of cobalt nitrate and 20 g of ammonium thiocyanate in 100 mL water.

2.2 Assay procedure:

A Pluronic F68 standard (200 mL), 100 µL cobalt thiocyanate reagent, 200 µL of ethyl acetate and 80 mL of absolute ethanol were mixed well in a microcentrifuge tube (1.5 mL) and then centrifuged for 1 min at 10,400 g. After centrifugation, the upper two layers were aspirated using a standard 200 µl pipette. Care was taken not to disturb the sediment. The sediment and tube walls were washed with 200 µL of ethyl acetate several times until the aspirated ethyl acetate became colourless. The sediment was dissolved in 2 mL acetone and the absorbance was measured by Pharmacia LKB-Ultrospec II spectrophotometer at 328 nm. A standard curve was constructed and the concentration of Pluronic F-68 in the unknown samples was determined accordingly. Absorbance measurement was carried out in triplicate for all the standard dilutions and unknown samples.

3. ELISA for EPO:

3.1 Materials: 96 multi-well plate was obtained from Fisher Scientific Ltd. Monoclonal anti-EPO antibodies were prepared in-house from a mouse/mouse anti-human EPO (5F12AD3) secreting hybridoma. All other chemicals were obtained from Sigma Canada.

3.2 Method:

All supernatant samples were analyzed using an ELISA that was developed in our lab. Briefly, polyclonal anti-human EPO antibody (4 μg/mL) in 0.1 M of sodium bicarbonate buffer was coated on a 96-well plate overnight. The plate was blocked with 3% BSA/PBS for 2 hours and then incubated with samples or EPO standards for eight hours at room temperature. Mouse monoclonal anti-EPO antibody diluted at 1:300 was incubated overnight at 4 °C. Alkaline-phosphatase conjugated with anti-mouse IgG (1:15,000 diluted in 1%BSA/PBST (PBS with 0.05% tween) was incubated for 4 hours at room temperature. The colour was developed by adding p-nitrophenyl phosphate substrate and incubating overnight at room temperature. The absorbance at 405 nm was measured by an ELISA reader. The plate was washed with washing buffer (PBST) four times after each step.

4. Hydrophobicity index assay:

4.1. Materials

Canola oil was obtained as Crisco (Procter & Gamble Inc. Toronto, Canada) and olive oil as Petrelli (Aldo Petrelli, Calgary, Canada). The manufacturers indicated that the canola oil contained traces of citric acid while the olive oil was 100% pure. Trypsin inhibitor

was obtained from GibcoBRL (Burlington, Canada). All other chemicals were purchased from Sigma. Centrifuge tubes were obtained from Corning Inc., N.Y., U.S.A.

4.2. Assay of hydrophobicity

A non-polar liquid (2 mL) was added to a 10-cm Kimax screw cap tube (Fisher Scientific, Canada). Exponentially growing cells were harvested by centrifugation (370xg) and re-suspended in PBS (supplemented with 2 mM glucose) to a cell density of ~ 10^6 cells/mL. An aliquot (2 mL) of the cell suspension was added to the Kimax tube. Immediately after the addition the tube was vortexed using a Baxter®Rot-mixer at speed mark 5 (50% of the maximum speed) for 10 seconds. The suspension was left for 5 min to allow for the two phases to separate. Then using a Glaspak Glass and a small gauge needle, a sample of the cell suspension in the lower aqueous layer was removed for counting. Care was taken to minimize liquid mixing during this stage. The viable cell concentration in this sample and in an untreated control sample was determined by haemocytometer counting following suspension in trypan blue. The hydrophobic index was determined as the proportion of the cell population that adhered to the hydrophobic phase by forming oil cell droplets in-between the two phases, the polar and aqueous. This was determined from the loss of viable cells from the aqueous phase.

$$\text{Hydrophobicity index} = \left[\frac{\text{Initial viable cell concentration in PBS} - \text{Final viable cell concentration in PBS}}{\text{Initial viable cell concentration in PBS}}\right] \text{X}100$$

4.3. Statistical Analysis:

The statistical significance of the results was determined by Analysis of Variance performed by SAS program, version 7.

5. Linoleic Acid uptake measurements:

5.1 Materials:

Radioactive ^{14}C linoleic acid was obtained from Amersham Pharmacia Biotech Inc. Baie d' Urfe, Canada. Gelman filter discs (0.2 μm) were obtained from Pall Canada Ltd, Mississauga, Canada.

5.2 Methods:

Reagent Preparation:

The linoleic acid was either complexed with bovine serum albumin (BSA), OR β-cyclodextrin, or was dissolved at high pH and then added to the medium. Radio-labelled (hot) ^{14}C linoleic acid (1 Ci/mole) was added by taking 270 μL of radiolabelled ^{14}C linoleic acid dissolved in toluene (equivalent to 27 μCi of radioactivity) and air drying it in 15 mL polypropylene tube. MilliQ water was added (3 mL) containing either BSA or cyclodextrin (10 mg/mL), or nothing. Non radio-labelled (cold) Linoleic acid (3 μL) was added from a stock solution in ethyl alcohol (1 M). The solution was mixed for 1 hour at room temperature. Adding 1mL of this preparation to 100 mL of medium gave a final concentration of 10 μM of "cold" linoleic acid and 1.8 μM of "hot" linoleic acid (total of 11.8 μM of linoleic acid).

Assay:

CC9C10 hybridoma cells (12 mL) at a density of 1.5 x 10^6 cells/mL were incubated at 37° C and continuously shaken with growth medium containing 11.8 μM linoleic acid. Samples of cells (0.5 mL) of well homogenously suspended cells were taken every 5 min and trapped on a Gelman disc filter (0.2 μm) by vacuum filtration. The cells were washed at least 4 times with ~ 5 mL BSA (0.1 %) in PBS. The radioactivity of the filters (with trapped cells) was measured using liquid scintillation counting.

6. Plasma Membrane Fluidity measurements:

Hybridoma CC9C10 cells (1.1 x 10^7 cells/mL) were suspended in 0.5 mL of PBS and incubated for 3 minutes with a dry film of 5-DOXYL-stearic acid while stirring on a shaker (Thermolyne Rotomix type 50800) with a speed setting of 0.5. The dry film was prepared by adding 10 μl of 5 mg/mL solution of 5-DOXYL-stearic acid in chloroform to a small glass tube and evaporating to dryness. The labelled hybridoma cells were centrifuged and the pellet was re-suspended in a 16 μL of PBS containing up to 0.2% Pluronic F68 for studying the effect of Pluronic F68, or PBS alone for studying the effect of linoleic acid. The 16 μL cell suspension was transferred to a sealed-end glass capillary tube. The spin spectra were measured by electron spin resonance (ESR).

ESR spectra were obtained using a BRUCKER EMX spectrometer with a modulation amplitude of 0.5 Gauss (G), modulation frequency of 100 kHz, microwave power of 19.87 mW and frequency of 9.3 or 9.4 GHz. Every spectrum was an average of 10 scans and the temperature was controlled at 37° C using nitrogen gas flow system. To analyse

the ESR spectra the hyperfine inner splitting ($A_\perp$) and outer splitting ($A_\parallel$) were obtained and used to calculate the order parameter (S) according to the equations (1) and (2) (Curatola et al. 1985; Foucher et al. 1996):

$$S = 0.564 \times (A_\parallel - A_\perp) / a' \quad \text{.....(1)}$$

$$a' = 1/3 \, (A_\parallel + 2 A_\perp) \quad \text{.....(2)}$$

8. Sensitivity to shear measurements:

8.1 Materials:

A cone-and-plate viscometer (Rheolab MC1) with MK91 measuring cone and 0.025 mm gap was obtained from Paar Physica, New Jersey, USA (Figure A.5 in the Appendix).

8.2 Method:

Prior to each experiment cells were taken from the spinner flask, centrifuged at 287 g for 5 min and then re-suspended in a protein-free, Pluronic-free, and bicarbonate-free medium. The absence of bicarbonate prevented an increase in the pH. The cell suspension was contained in a vial stirred in a water-bath and maintained for a fixed period of time at 37° C unless otherwise mentioned. After 10 to 40 min incubation 5.5 mL of a cell suspension with a density of 10^6 cells/mL was subjected to a high shear rate of either 11,000 s^{-1} or 12,000 s^{-1} in a MC1 cone and plate viscometer. The temperature was controlled at either 37° C or 22° C by circulating water through the plate section of the viscometer. In test samples, Pluronic F68 was added at least 5 min prior to the application of the shear force

9. Viscosity Measurements:

Rheolab MC1 viscometer was used to measure the viscosity of growth media and the results were compared to the viscosity of distilled water at 22 °C. All measurements were made at shear rate of 10 000 s^{-1} for 2 min. The viscosity was calculated by dividing the shear rate (τ) over the shear stress (γ), ($\tau=\gamma^*\beta$) where β is the viscosity (n=20).

Chapter 3 : CHO growth medium optimization

Serum free media are preferred over animal serum supplemented media because they are (a) defined, (b) more consistent, (c) make the purification of released proteins easier and (d) help avoid animal borne infective particles that are difficult to screen for. Therefore serum free medium was the choice to grow CHO cells.

APOsfm 1.1 is a serum free medium that was developed by Cangene® Corporation. This medium gave an inconsistent cell growth rates and high degree of cell clumping which affected the cell growth and cell counting. The medium was passed to Prof. M. Butler's laboratory group for further optimization. During the process of optimization the name of the medium was changed from APOsfm 1.1 to APOsfm 2.0 and finally to CNJsfm 2.1.

Cangene® Corporation has produced two clones of CHO-EPO cells by transfecting CHO-K1 cells with the human Erythropoietin (EPO) gene. The first clone is designated EPO-2-4-84* (called in the thesis as CHO 84*) and was transfected in Ex-Cell® 301 medium (a proprietary medium by JRH), while the other clone was EPO-2-5-81 (called in the thesis as CHO 81), which was transfected in APOsfm 1.1 medium. The EPO-2-4-84* clone became unstable upon transfer from Ex-Cell® 301 to APOsfm 1.1 medium and over extensive period it lost its ability to produce EPO. On the other hand EPO-2-5-81 cells gave consistently higher yield of EPO but the cell growth was slow.

The objective of this chapter is to describe the optimization of APOsfm 1.1 medium to achieve consistent cell growth and high EPO productivity. The optimization of the medium was a team effort, which involved everyone in the laboratory. Therefore, results discussed here are only a portion of this teamwork. The effect of additives like Ex-Cyte®, Cholesterol, α-Tocopherol, Cobalt Chloride, and hypoxanthine with thymidine were tested. As a result it was decided to supplement the medium with hypoxanthine and thymidine and to remove Ex-Cyte® to achieve better cell yield and EPO production. The final medium was called CNJsfm 2.1 medium and was utilized for all subsequent CHO cell culture in this thesis.

1. Effect of Excyte:

The need for a source of lipids in serum-free media has been suggested and reported in many citations in the literature (Iscove et al. 1984; Bjare 1992). However, lipids are insoluble in water and the method of introduction into solution has an important effect on their ability to support cell growth (Gorfien et al. 2000). Ex-Cyte VLE (Very Low Endotoxin) is a water-soluble lipid extract from bovine serum. It is an undefined component that contains different saturated and unsaturated fatty acids, lipopotein, phospholipids and cholesterol. Table 3.1 shows the composition of Ex-Cyte supplement (Serologicals Inc.).

Ex-Cyte was a component in APOsfm 1.1 and it was added as 0.25% (v/v). However later experiments in our laboratory showed an inhibitory effect of this concentration (Yang 2001). Therefore the concentration was decreased to 0.06%. The difference

between 0 and 0.06% was marginal therefore an experiment was set up to determine whether this supplement added any beneficial effect on this clone of CHO cells or not.

Table 3.1. Lipid Composition of EX-CYTE® VLE. Serologicals Inc.

	mg/mL
Cholesterol	9.40
Triglycerides	0.07
Phospholipids	
Lecithin	4.12
Lysolecithin	0.95
Sphingomyelin	2.95
Phosphatidylethanolamine	0.02
Total	8.04
Total Fatty Acids	9.60

Table 3.2 shows the effect of Ex-Cyte® VLE on the growth of CHO 84* cells. APOsfm 2.0 contains 0.06% (v/v) of Ex-Cyte® VLE while the Control is Ex-Cyte® free. It can be seen that Ex-Cyte® VLE has a significant inhibitory effect on the growth of CHO cells. The P value for the paired T test was < 0.001. The coefficient of variance (C.V.%) was 20.5% for the control and 34.3% for APOsfm 2.0. This shows that the consistency of cell yield was better with cells cultured in the absence of Ex-Cyte®. On the other hand, from the morphology point of view clumping was higher in the presence of Ex-Cyte®, which might be a factor in the decrease of the cell yields. Big clumps may contain an inner core in which cells are deficient in nutrients and dissolved oxygen leading to a decrease in viable cell yield.

Table 3.2 Effect of Excyte on the growth and EPO production of CHO 84* cells.

	Control ± (S.E.M)	+ Excyte ± (S.E.M)	T-Test (p)
Cell Yield (x 10^5 cells/mL)	5.94 ± 0.39	4.65 ± 0.5	0.001
EPO production (IU/mL)	132 ± 16.7	82 ± 5.9	0.017
Specific EPO production ($IU/10^6$ /day)	98.3 ± 14.3	78.1± 6.7	0.020

The EPO production is significantly lower in the presence of Ex-Cyte® VLE. The specific EPO productivity of these cells has also decreased significantly reflecting a negative effect on the synthesis and/or the secretion of this glycoprotein.

Mammalian cells in culture have been reported to require lipids for growth to build a robust cell membrane or produce energy. However, cells in culture lack a controlling mechanism to regulate lipid absorption. Free fatty acids absorption, for example has been shown to follow passive diffusion behaviour. Therefore any unwanted fatty acids might be taken up into the membrane producing a negative effect on cell growth or productivity. Uptake of an excess of fatty acids in culture has been reported to lead to vesicle formation, which might be a cause of decreased cells growth and recombinant protein production (Butler 1995). On the other hand, selective additions of cholesterol or fatty acids like linoleic acids might be a more effective method in promoting cell growth or EPO production since the concentration of each and every component can be tested and optimized separately.

Ex-Cyte was reported to increase the cell growth and productivity of many cell lines including CHO cells (Jenkins et al. 1994) and insect Sf-9 cells (Hink et al. 1991). However, the experimental conditions performed by Jenkins et al. (1994) were different from those reported here. All cell culture experiments on Ex-Cyte® VLE here were performed in stationary T-flasks while Jenkins et al. (1994) performed their experiments in agitated flasks. This might have a significant effect since lipids promote clumping (our unpublished observations), which is more evident in stationary culture than in agitated ones. Furthermore, the difference between different CHO transfected cells is very important since different recombinant proteins require different nutritional factors.

Therefore the conclusion was to remove Ex-Cyte® from the medium.

2. Effect of Hypoxanthine and Thymidine (HT):

Hypoxanthine and Thymidine are DNA precursors during the S-phase of the cell cycle. Mammalian cells can synthesize their own nucleotides, however, adding hypoxanthine and thymidine in the medium has been reported to increase the cell growth rate (Rothblast 1971). We tested the effect of adding hypoxanthine and thymidine in concentration of 10 and 2 μg/mL for hypoxanthine and thymidine respectively on two clones CHO 84* and 81 cells. Cells were cultured in 25 cm^2 T-flasks and were passaged for 8 subsequent passages.

Table 3.3 shows the results for the effect of hypoxanthine and thymidine addition on the growth of 84* in CNJsfm 2.1 medium (n=8). There was a significant increase in cell

yields after 4 days of culture with a mean increase of 0.91 x 10^5 cells/mL. EPO production was not significantly different in the presence of HT.

Table 3.3 Effect of HT on the growth and EPO production of CHO 84* clone.

	Control	HT	T-Test (p)
Cell growth (x10^5 cells/mL)	5.9 ± 0.6	6.8 ± 0.4	0.011
EPO production (IU/mL)	140 ± 20	149 ± 25	0.67
Specific EPO production (IU/10^6 cells/day)	109 ± 9	100 ± 13	0.41

With this slight but significant increase in the cell yield of 84* cells, two other CHO cells were tested, the 81 clone and the CHO parent cells. The experimental set up was the same as above. Table 3.4 shows the effect on 81 cells. Results show a consistent and a significant increase in CHO 81 cell growth with HT addition (n=8). There is also a consistent and a significant increase in EPO production with HT addition. The specific productivity of EPO did not increase significantly. This is probably because the altered growth rate was not sufficient to show a significant difference in the specific productivity. Hypoxanthine and thymidine stimulated the growth of parent cells.

To optimize the concentration of this combination of additives, a range of concentrations from 2.5 to 40 μg/mL and 0.5 to 8 μg/mL hypoxanthine and thymidine respectively were tested. The ratio of 5:1 hypoxanthine to thymidine has been kept at all times. All cultures were set in stationary 25 cm^2 and were cultured for 4 days.

Table 3.4 The effect of HT (10/2 μg/mL) on the CHO 81 clone and parent cells.

	Control	HT	T-Test (p)
Yield of 81 clone ($x10^5$cells/mL)	6.7 ± 0.3	7.53 ± 0.3	<0.001
EPO production (IU/mL)	210.1 ± 23	311.2 ± 14	<0.001
Specific EPO production ($IU/10^6$ cells/day)	92.6 ± 19	201.3 ± 11	0.37
Yield of Parent cells ($x10^5$cells/mL)	10.9 ± 0.6	13 ± 0.9	0.05

Figure 3.1 shows the effect of different concentrations of HT on the growth and the EPO productivity of CHO cells in stationary T-flasks (n=2). There is a gradual increase in cell growth and subsequent EPO production with increasing concentrations of HT. There is no significant difference between 10/2, 20/4 and 40/8 (Hypoxanthine/Thymidine μg/mL) concentrations on cell growth while 10/2 gave the highest produced EPO. Higher concentrations showed inhibition for EPO production, therefore 10 μg/mL of hypoxanthine and 2 μg/mL thymidine are the optimal concentrations for both cells growth and EPO production. Figure 3.2 shows the effect of HT on the growth and EPO production of CHO cells in spinner flasks at 45 rpm (n=2). Cells were cultured in 100 mL spinner flasks and samples were taken daily for cell density and EPO concentration analysis. The results show a significant increase in both cell yield and EPO production over 10 days.

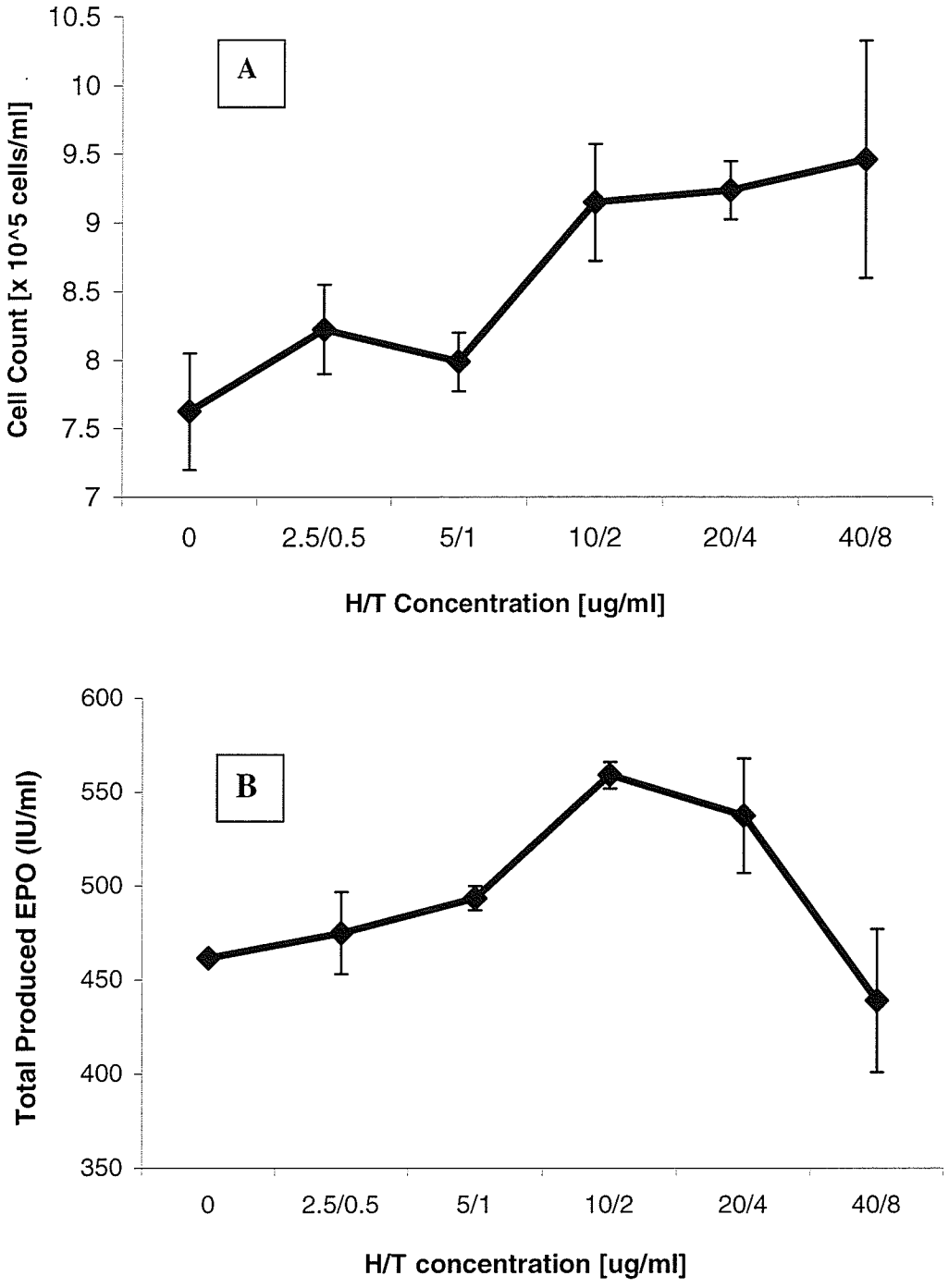


Figure 3.1 Effect of Hypoxanthine and Thymidine (HT) on the growth and EPO production of CHO 81 cells.

Error bars represent the cells yield or EPO production of each trial (n=2). Cells were cultured in 2x 25 cm^2 T-flasks for 4 days. Inoculation density was 10^5 cells/mL. (A) Final cell yield and (B) is total EPO production data

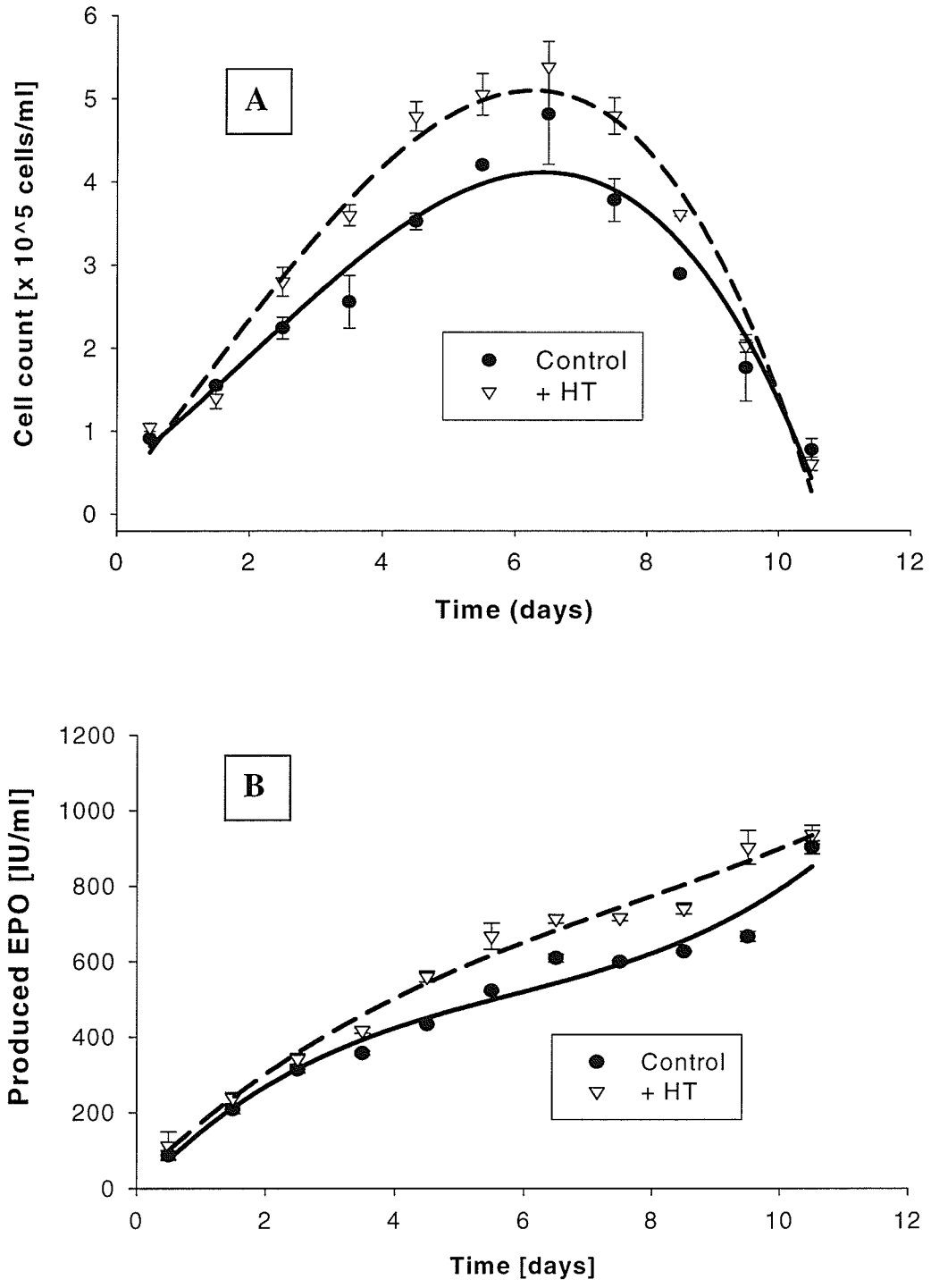


Figure 3.2 Effect of Hypoxanthine and Thymidine (HT) on the growth and EPO CHO 81 cells in spinner flasks at 45 rpm.

Error bars represent the cells yield of each trial (n=2). Cells were cultured in 100 mL spinner flasks with inoculation density of 10^5 cells/mL for 10 days. (A) Cell growth data while (B) is EPO production data.

Hypoxanthine and thymidine are DNA precursors used by mammalian cells for DNA synthesis during the S-phase of the cell cycle. However, cells can synthesize their own DNA precursors from simple amino acids like glycine, aspartate, and glutamine in the presence of tetrahydrofolate *(the de novo synthesis)*, which in turn needs the DHFR enzyme. They can also utilize hypoxanthine and thymidine provided in the medium (*the salvage pathway*). In the latter case hypoxanthine-guanine phosphatidyl transferase (HGPT) enzyme is needed (Yamaoka et al. 1997).

In this thesis, the CHO cells were DHFR positive and therefore are expected to synthesize their own nucleotides. Despite this, the addition of hypoxanthine and thymidine were found to increase the cell growth. There are two possible reasons that could explain this phenomenon. One is that the transfected CHO cells have lost some DHFR activity and therefore they needed an external source of DNA precursor. Alternatively, the cells could have used the extra source of hypoxanthine and thymidine to save energy for cell growth and recombinant protein production. Non-transfected CHO parent cells responded positively to HT with a significant increase in cell yield (Table 3.4). This finding supports the second explanation and agrees to what was reported before that hypoxanthine and thymidine improve growth rates of DHFR positive cells (Rothblast 1971). It was also reported that the salvage pathway inhibits some of the activity of the de novo pathway to save energy when both pathways are working (Yamaoka et al. 1997).

The decision was taken to include HT in the medium.

3. Effect of Cholesterol

Cholesterol is a large molecule that is embedded in the lipid bilayer of the cell membrane and its presence is required for the optimal fluidity and robustness of the cell membrane. Cholesterol has a very important regulatory role in phospholipid/protein membrane permeability. Its incorporation into the cell membrane phospholipid bilayer can help modulate the membrane arrangement of specific proteins (Papahadjopoulos et al. 1973).

Several papers in the literature have reported an increase in the cell growth and recombinant protein production of CHO cells using cholesterol concentrations between 1 to 20 μg/mL (Castro et al. 1996; Gilbert et al. 1996). However others reported an inhibitory effect on CHO cell growth (Castro et al. 1996). Therefore we tried several concentrations of cholesterol on the two transfected clones in stationary and in agitated cultures. Concentrations of 10 and 5 μg/mL were tried on the CHO 84* clone in stationary T-flasks while 0.45 μg/mL was tried on the CHO 81 clone in both stationary and agitated cultures. Cells were cultured in 25 cm^2 T-flasks for total of 8 consecutive passages. Cholesterol was dissolved in ethyl alcohol for the 10 and 5 μg/mL concentrations while cyclodextrin complexed cholesterol (Sigma) was used for 0.45 μg/mL. Table 3.5 shows the effect of these concentrations on CHO 84* and CHO 81 cells. The results here show that a concentration of 10 μg/mL was inhibitory for cell growth and EPO production. Cell yields and EPO productivity were lower with cholesterol compared with control. The difference is statistically significant ($p < 0.05$). There was also a decrease in the specific EPO productivity, which suggests an interaction with the EPO secretion or synthesis pathways. On the other hand in Table 3.5 results

show that lower concentration of 5 μg/mL had no significant effect on both cells yields and EPO production. The T-test indicates a non-significant difference ($P > 0.05$) between cultures with cholesterol compared with the control.

Table 3.5 Effect of Cholesterol on the growth and EPO production CHO cells in T-flasks.

	Control ± (S.E.M)	+ Cholesterol ± (S.E.M)	T-Test
A) Effect of Cholesterol (10 μg/mL) on CHO 84* cells.			
Cell Yield (x 10^5 cells/mL)	5.86 ± 0.55	5.08 ± 0.43	0.02
EPO production (IU/mL)	139.5 ± 19.5	74.0 ± 14.4	0.03
Specific EPO production (IU/10^6 /day)	109 ± 8.5	66.3± 9.33	0.03
B) Effect of Cholesterol (5 μg/mL) on CHO 84* cells.			
Cell Yield (x 10^5 cells/mL)	5.86 ± 0.55	5.56 ± 0.40	0.28
EPO production (IU/mL)	139.5 ± 19.5	149.4 ± 22.5	0.38
Specific EPO production (IU/10^6/day)	109 ± 8.5	156.0± 14.3	0.23
C) Effect of Cholesterol (0.45 μg/mL) on CHO 81 cells.			
Cell Yield (x 10^5 cells/mL)	6.12 ± 0.54	6.15 ± 0.40	0.94
EPO production (IU/mL)	291.9 ± 28.7	292.6 ± 29.2	0.47
Specific EPO production (IU/10^6 /day)	176.9 ± 32.2	167.5 ± 28.3	0.39

Using lower concentration of 0.45 μg/mL with another clone (CHO 81) did not make the situation better. Cholesterol again did not show any statistically significant difference on cell growth or EPO productivity in cell growing in stationary T-flasks as shown by the T-test (p value > 0.05).

On the other hand, Figure 3.3 shows the effect of 0.45 μg/mL of cholesterol on the CHO 81 clone in agitated cultures in spinner flasks at 45 rpm. Cell growth was statistically higher (Figure 3.3a) than the control. However there was no difference in the amount of produced EPO at both the 5 and 9 days culturing periods (Figure 3.3b).

Cholesterol exists in cells in two forms: as free cholesterol, which is a required structural component for all mammalian plasma membranes, and as an esterified cholesterol, whose only known function is to serve as a storage form of free cholesterol. Both forms are insoluble in water and require a hydrophilic carrier molecule. The method of introduction into solution has a significant effect on its ability to support cell growth. Cholesterol can be dissolved in alcohol and then introduced into the medium or water-soluble form of cholesterol can be used. The water-soluble cholesterol is usually complexed with a carrier molecule like cyclodextrin. GIBCO of Life Technologies has reported that they have two forms (1^{st} generation and 2^{nd} generation) of cholesterol/cyclodextrin complex. The difference is based on the complexation ratio between the two molecules (cholesterol and cyclodextrin). Gorfien et al. (2000) has shown that there was a significant effect of using the 2^{nd} generation (proprietary for Gibco) cholesterol supplement compared with 1^{st} generation cholesterol supplement to support the growth of NS0 cells (a cholesterol

auxotroph). LT1 1st generation cholesterol supplement has been shown to be trapped in the filters during the sterilization process while around 70% of the supplement was able to pass the filter in the LT1 2nd generation cholesterol supplement. Therefore cells might show a different effect than found if another way of complexing cholesterol was used or different ratios of cholesterol/cyclodextrin were used. This might explain the difference in the results obtained here with the findings elsewhere showing a beneficial effect of cholesterol for CHO cells. Alternatively the difference could be due to differences in the clone or the recombinant protein used.

3.2 Cholesterol with HT

Hypoxanthine, thymidine and cholesterol all are produced by the cells but their synthesis require a considerable level of energy through usage of ATP. Therefore adding these additives together might save some energy and might give cells an advantage in producing recombinant proteins. An experiment was performed in stationary T-flasks as well as agitated spinner flasks to determine the effect of cholesterol, hypoxanthine and thymidine combined. The results showed significantly higher cell growth in both T-flasks and agitated cultures (T-test, $P < 0.01$). The average cell yield for control cells in T-flasks was 4.95 ± 0.35 while with hypoxanthine, thymidine and cholesterol (HTC) the cell yield was 8.23 ± 0.85 (x 10^5 cells/mL ± SEM, n=5). Similar results were found in spinner flasks (Figure 3.3) but even with the higher cell densities obtained the total EPO produced did not increase showing a significant decrease in the specific productivity of these cells (Figure 3.3).

The conclusion was NOT to add cholesterol

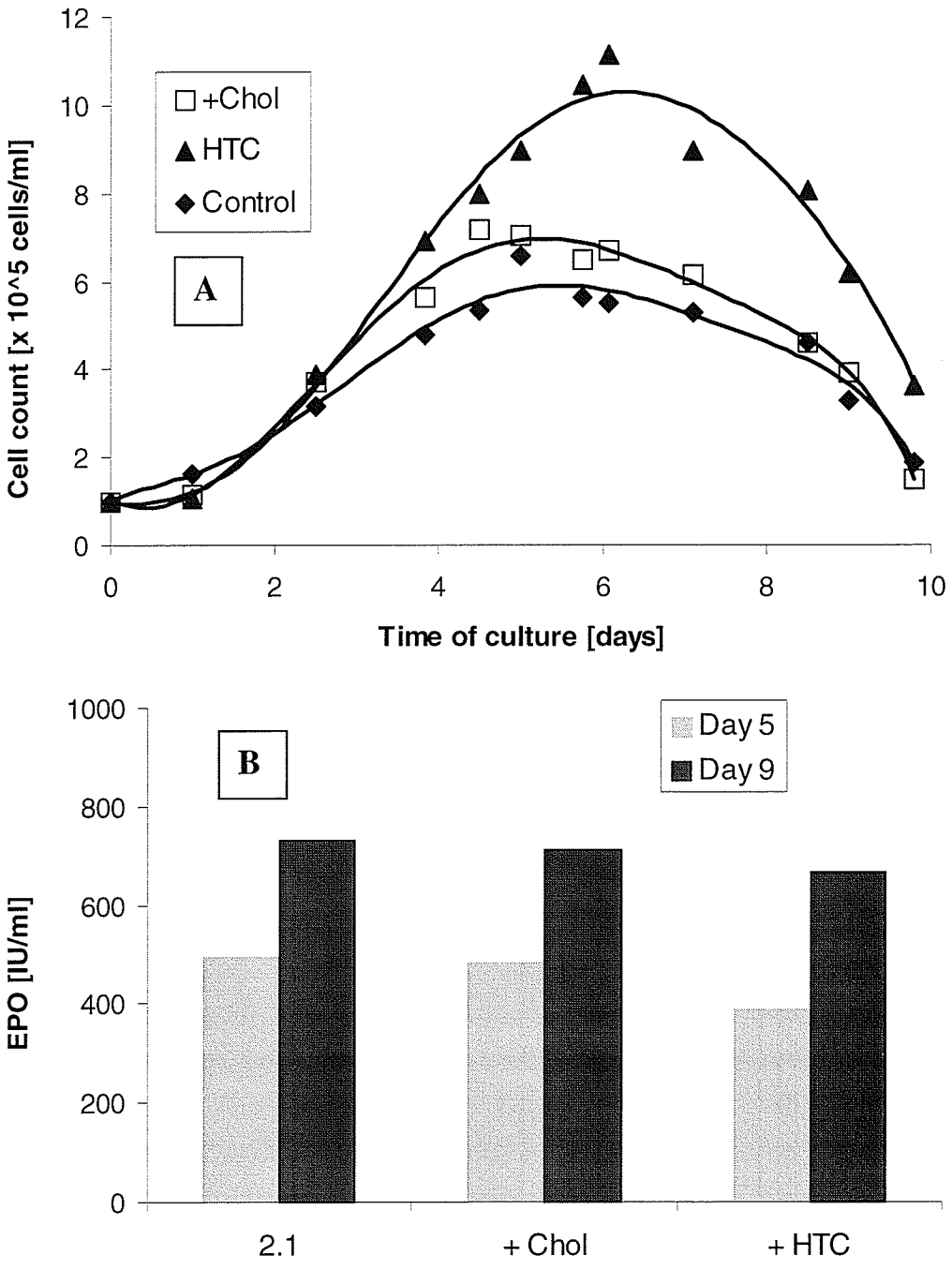


Figure 3.3 Effect of Hypoxanthine, Thymidine and Cholesterol (HTC) combined on the growth and EPO production in CHO 81 cells in spinner flasks at 45 rpm.

Cells were cultured in 100 mL spinner flasks with inoculation densities of 10^5 cells/mL for 10 days (n=1). (A) Cell growth data while (B) is EPO production data. +Chol = Cholesterol added (0.45 µg/mL) only.

4. Effect of α-Tocopherol

α-Tocopherol is an anti-oxidant vitamin that is important for many mammalian cells. It works as a scavenger for oxidant radicals that might react with cell membrane lipids affecting the function of these membranes and the robustness of the cells. This vitamin has been reported to increase the survival of hippocampal and striatal neurons in dissociated culture (Sato et al. 1993) and protect the human fibroblasts and rat skin cells from the oxidative damage of UVA radiation (Ezra et al. 1996). Therefore we tested the effect of α-tocopherol on the growth rate and EPO production of CHO cells on both 84* and 81 clones in stationary 25 cm^2 T-flasks (n=8).

Table 3.6 shows the effect of tocopherol addition on the growth and EPO production of CHO 84*. No significant effect could be seen with the addition of α-tocopherol on the growth of CHO 84* cells, while the EPO production was significantly higher with α-tocopherol addition. There was no significant effect on either growth or EPO production of CHO 81 cells with cell yield of 6.15 ± 0.54 x 10^5 cells/mL and EPO production of 382.8 ± 70.5 IU/mL in the presence of α-tocopherol. The lack of effect of α-tocopherol could be due to the presence of other serum proteins like fetuin or BSA. Chepda et al. (1999) has showed that this vitamin is consumed rapidly in protein free cultures, which did not happen in the presence of serum proteins like BSA.

There was a difference in the effect of tocopherol on the EPO production of CHO 81 and CHO 84* clones. The difference in the response of these two clones can be due to the difference in the basal level of EPO production in each clone.

Table 3.6 Effect of Tocopherol on the growth and EPO production of CHO 84* clone.

	Control ± (S.E.M)	Tocopherol ± (S.E.M)	T-Test
Cell Yield (x 10^5 cells/mL)	3.48 ± 0.65	5.39 ± 0.52	0.57
EPO production (IU/mL)	34.4 ± 11.5	106.8 ± 18.13	<0.001
Specific EPO production ($IU/10^6$ /day)	31.6 ± 9.3	85.3 ± 15.3	<0.001

Therefore because there was no significant effect on CHO 81 cells we concluded Not to add α-tocopherol.

5. Cobalt Chloride

Cobalt is one of the trace elements that have been reported to support CHO cell growth (Messi 1991). It was also reported to promote the attachment and decrease clumping of Pheochromocytoma (PC12) cells (Lin et al. 1993). This effect was specific for cobalt chloride and was not seen with other di-valent cations with the exception of manganese. Cobalt chloride was supplemented to the media at a concentration of 0.1 mg/L, CHO 81 cells were cultured in 25 cm^2 flasks for 8 passages and the results were averaged. Table 3.7 shows these results on 84* cells. Cobalt chloride addition did not show any significant effect on the growth of 84* CHO cells, but enhanced the EPO production significantly. On the other hand there was a slight but significant inhibition on the growth of 81 cells with no effect on EPO production. The average cell yield was 5.82 ± 0.5 x 10^5 cells/mL and the total produced EPO was 353.0 ±66.

The reason for the difference between the results reported here on CHO cells and the ones reported by Messi (1991) could be the different clone we are using. The reason could also be the concentration used, which might not be the optimal one.

Table 3.7 Effect of Cobalt Chloride ($CoCl_2$) on the growth and EPO production of CHO 84* cells (n=8).

	Control ± (S.E.M)	CoCl2 ± (S.E.M)	T-Test
Cell Yield (x 10^5 cells/mL)	5.34 ± 0.65	5.28 ± 0.53	0.47
EPO production (IU/mL)	34.4 ± 11.4	108.7 ± 15.9	0.01
Specific EPO production ($IU/10^6$ /day)	31.6 ± 9.3	90 ± 5.5	<0.001

Therefore we concluded Not to add cobalt chloride because of no significant effect on CHO 81 cells.

6. Conclusions:

- Ex-Cyte® was removed from the medium because of its inhibitory effect on cell growth and EPO production.
- HT was added in the medium because of its positive effect on cell growth and EPO production.
- Cholesterol was not added because of no significant positive effect on EPO production at any of the tried concentrations.
- α-Tocopherol was not added because of no positive effect on CHO 81 cell growth or EPO production.

- Cobalt chloride was not added because of no significant effect on CHO 81 cells growth and EPO production.

Chapter 4 ♣: The Effect of Pluronic F68 in the presence of bubbles

The ability of Pluronic F68 to minimize the detrimental effects of bubbles is now well recognized in the literature. It is also accepted that decreasing cell to bubble attachment is the key mechanism of Pluronic F68 in ameliorating the damaging effects of bubbles (Zhang et al. 1992). However it is unknown how Pluronic does that. It is suggested that Pluronic F68 can saturate the bubble surface and thereby change the surface property of bubbles to prevent their attachment to the cells (Jordan et al. 1994). Others suggest that Pluronic F68 can change the surface hydrophobicity of cells making them less reactive with bubble surfaces (Wu et al. 1997).

In this chapter the protective effect of Pluronic F68 during bubble bursting at the air-liquid interface on the top portion of the culture is further investigated. An assay was developed to measure the hydrophobicity of mammalian cells and used to study the effect of Pluronic F68 on this measurement in three well known cell lines. Furthermore, Pluronic F68 was compared with bovine serum albumin (BSA) as an alternative additive that may protect cells from bubble damage.

1. Assay development

The purpose of the assay described in this chapter is to provide an index of measurement of the hydrophobic interactions of mammalian cells grown in culture. The assay was

♣ The content of this chapter was published in: Ghebeh, H. Gillis, J. Butler, M. (2002) Measurement of hydrophobic interactions of mammalian cells grown in culture. *Journal of Biotechnology* **95**: 39-48.

modeled on a method previously described for bacteria referred to as the BATH test (bacterial adhesion to hydrocarbons), which depends upon the partition of cells between two liquid phases (Rosenberg et al. 1980). The BATH test involves the measurement of the change of turbidity of an aqueous suspension following mixing with xylene, octane or hexadecane. However, this method cannot be applied directly to mammalian cells for at least two reasons. Firstly, mammalian cells are generally grown in suspension at much lower cell densities than bacteria and consequently turbidity is not a good measurement of changes of cell density of mammalian cells. Secondly, some of the non-polar solvents used in the BATH test are potentially damaging to the more fragile mammalian cells.

The haemocytometer counting method was adopted following suspension of cells in trypan blue (Philips et al. 1973). This is a commonly used method for determining the concentration of viable mammalian cells in suspension. This method has the advantage of distinguishing freely suspended from clumped cells and allows the determination of cell viability by the exclusion of the dye. In order to reduce the risk of cell damage, naturally occurring oils (olive oil and canola oil) were used either on their own or in combination with a hydrocarbon solvent.

A critical part of the assay is to start with a sufficiently high cell concentration in PBS. This was determined to be around 10^6 cells/mL. This minimal concentration is required in order to ensure an adequate measurement of differences in cell concentration before and after partitioning of the cells between the liquid phases. The hydrophobicity index was determined from the loss of cells from the aqueous suspension after vortexing with

an equal volume of a non-polar liquid (Table 4.1). A number of non-polar liquid phases were tested in this assay, and relatively consistent and measurable data with olive oil, canola oil or an olive/canola/hexadecane mixture were obtainable. Table 4.1 shows measurements of the hydrophobicity indices obtained from CHO cells harvested under different conditions and using these non-polar solvents. Data show that there were significant differences in the values obtained depending upon the non-polar phase used but also depending upon the method of treating the cells. Three alternative treatments are described as independent trials (A, B and C) in Table 4.1.

Table 4.1 Hydrophobicity index (%) of CHO 81 cells using three types of oil phases (n=3).

Trial	Olive oil	Canola oil	5/2/3 mix ♣
A	93.1 ± 5.7	91.6 ± 7.2	96.6 ± 2.8
B	62.1 ± 4.1	75.8 ± 4.0	73.0 ± 4.1
C	77.3 ± 2.3	81.7 ± 2.0	89.1 ±0.8

♣ (canola/hexadecane/olive v/v/v)

In trial A shown in Table 1, CHO cells were trypsinized from the growth medium prior to re-suspension in PBS. The purpose of the trypsinization was to free adherent cells from monolayer and to free cells from clumps that can develop in stationary culture. Using this protocol, relatively high values of the hydrophobicity index were obtained with each of the three non-polar solvents indicated. Apparently lower values were obtained when the cells were not trypsinized prior to analysis (trial B).

In both trials A and B the solutions were left to stand for up to 10 min following the addition of the cell suspension to the oil phase and before vortexing. In trial C the

mixture was vortexed immediately upon mixing the two liquid phases. This had the effect of increasing the apparent hydrophobicity index (compared to trial B). This was likely to be due to the fact that fewer cell clumps developed in the aqueous solution. The formation of cell clumps during the standing period in trials A and B could well have led to an underestimation of the cell concentration. These clumps did not form if the mixture was vortexed immediately. Another effect of the standardization of the time of vortexing was to reduce significantly the variability between replicates, as noted by the lower standard errors as shown in the results from trial C. In all the above trials the cells were left for 5 min to settle after vortexing as indicated in the Methods section.

As a result of these trials, it was decided to vortex the cell suspension for 10 sec immediately after addition of an equal volume of the oil and to use non trypsinized CHO cells cultured in suspension. This was adopted as a standard protocol for all subsequent assays. The potential physical damage to the cells by this procedure was determined by vortexing a cell suspension in PBS under identical conditions but with no oil phase. This resulted in a reduction of the viable cell concentration by <7% (n=10).

2.0. Effect of Pluronic F68 on the hydrophobic interaction of CHO cells

Pluronic F-68 is a non-ionic surfactant that is used in large-scale stirred-tank bioreactors of mammalian cell cultures to prevent damage of cells from stirring and gas sparging. Pluronic F68 as a surfactant has both hydrophobic and hydrophilic moieties and is more concentrated in the air-liquid interface around bubbles or close to the top headspace of culture vessels. The possibility that Pluronic F68 changes the hydrophobic interaction of

cells was investigated by the two-phase assay using the standard conditions that are described in Materials and Methods. Pluronic F68 was added to aliquots of a CHO cell suspension in PBS over a concentration range up to 0.2% (w/v). Pluronic F68 is commonly added to standard growth medium at a concentration of 0.1% in large-scale culture (Butler 1990).

Figure 4.1 shows the hydrophobicity index of CHO 81 cells measured at different concentrations of Pluronic F68. The experiment was repeated using five different non-polar solvents or mixtures. The results show that there was a significant decrease in the hydrophobicity index with an increase in the concentration of Pluronic F68. The hydrophobic index was at a minimal value at a Pluronic F68 concentration of 0.2% in the assay irrespective of the non-polar phase used. However, there were some significant differences in the values dependent upon the non-polar solvent used. At 0.1% and 0.2% Pluronic F68 the hydrophobicity indices were significantly lower in the assay using either hexadecane or canola oil. The index decreased to a value of zero at 0.2% Pluronic with either of these two non-polar phases. An index of zero indicates that there was the same concentration of viable cells in the aqueous phase before and after vortexing with the non-polar solvent.

The results in Figure 4.1 indicate that there was a significant advantage in the use of canola oil or hexadecane rather than the other solvents in the assay because of an increased sensitivity that maximized the difference in values obtained between the untreated and Pluronic-treated cells.

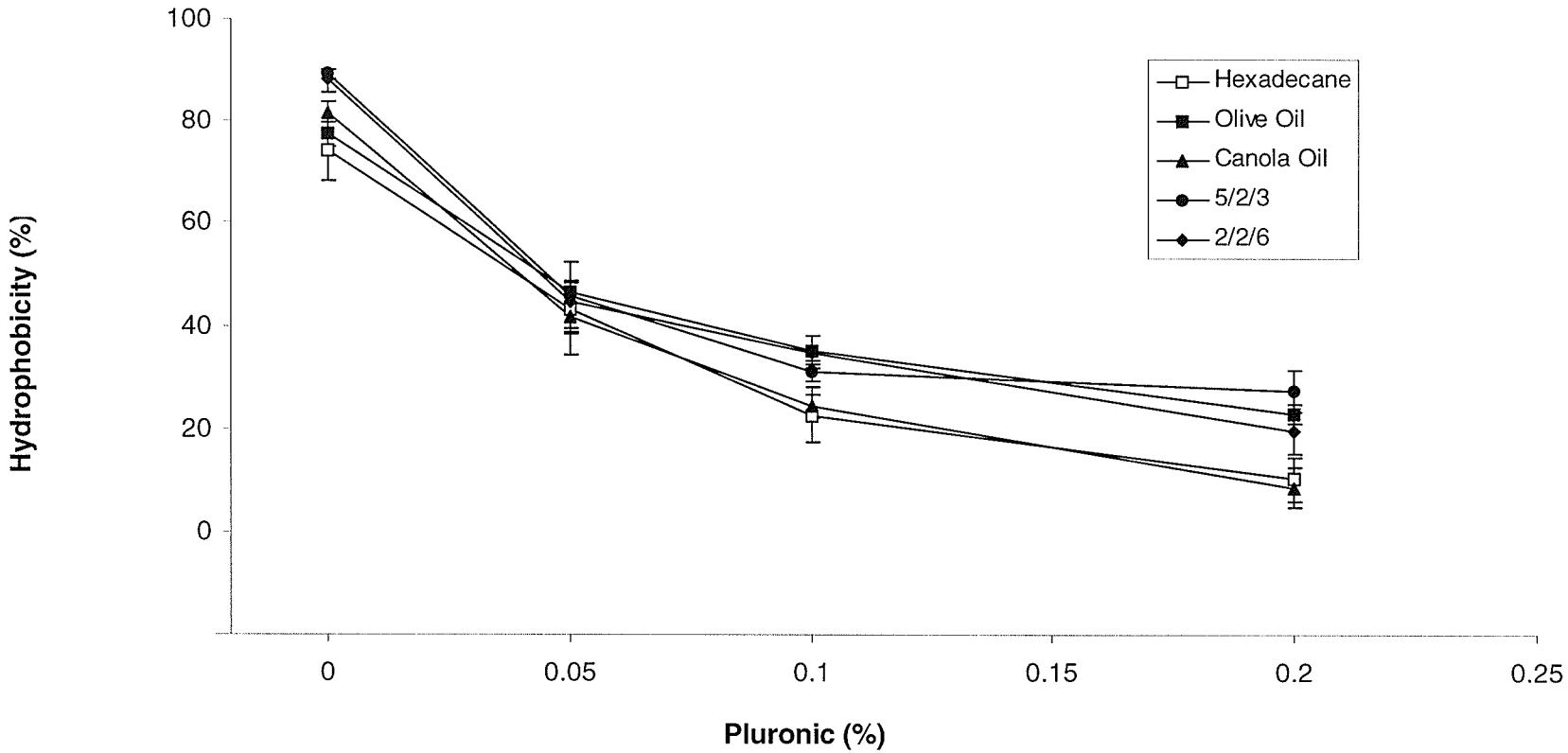


Figure 4.1 The effect of Pluronic F68 on the hydrophobicity index of CHO cells.

CHO cells at a density of 10^6 cells/mL were taken from growth medium and re-suspended in PBS. Pluronic F68 at concentrations up to 0.2% were added to 2 mL aliquots of this cell suspension. The cell surface hydrophobicity was determined at each Pluronic F68 concentration using the hydrophobicity assay under standard conditions as described in the Materials and Methods. Determinations were repeated five times with different non-polar liquid phases – hexadecane, olive oil, canola oil and mixtures of the three at 5/2/3 and 2/2/6 (canola/hexadecane/olive v/v/v). Each point is a mean with an error bar (±SEM) where number of repeats (n) =4.

3.0 Effect of Pluronic F68 on the hydrophobicity index of hybridoma cells and SP2/0 myeloma cells

Figure 4.2 shows the effect of Pluronic F68 on the hydrophobicity index of CC9C10 hybridomas. This assay was performed in the same way as for the CHO cells using the same 5 non-polar solvents and a Pluronic F68 concentration range up to 0.2% (w/v). In this case there was no significant difference in the profiles obtained using the different non-polar solvents. The initial hydrophobicity indices ranged from 45 to 64, depending on the non-polar solvent but in all cases a minimum index was obtained following the addition of 0.05% Pluronic.

There was a significant difference in the results obtained from the hybridoma and CHO cells (Figures 4.1 and 4.2). The hydrophobicity index of the untreated cells was significantly lower for the hybridomas. Furthermore, a lower concentration of Pluronic F68 was required to reduce the hydrophobicity index to zero. These results suggest that for bioreactor cultures the protective effect of Pluronic F68 for hybridomas may be offered at a concentration of 0.05% as opposed to 0.2% for CHO cells.

The effect of Pluronic F68 on SP2/0 myeloma cells was performed in an assay using canola oil as the non-polar phase (Figure 4.3). These results were similar to those of the hybridoma cells (Figure 4.2) in so far as a concentration of 0.05% Pluronic F68 reduced the hydrophobicity index of the cells to a minimal level. Also, the initial hydrophobicity index determined for the untreated cells (70) was close to the value determined for the hybridoma.

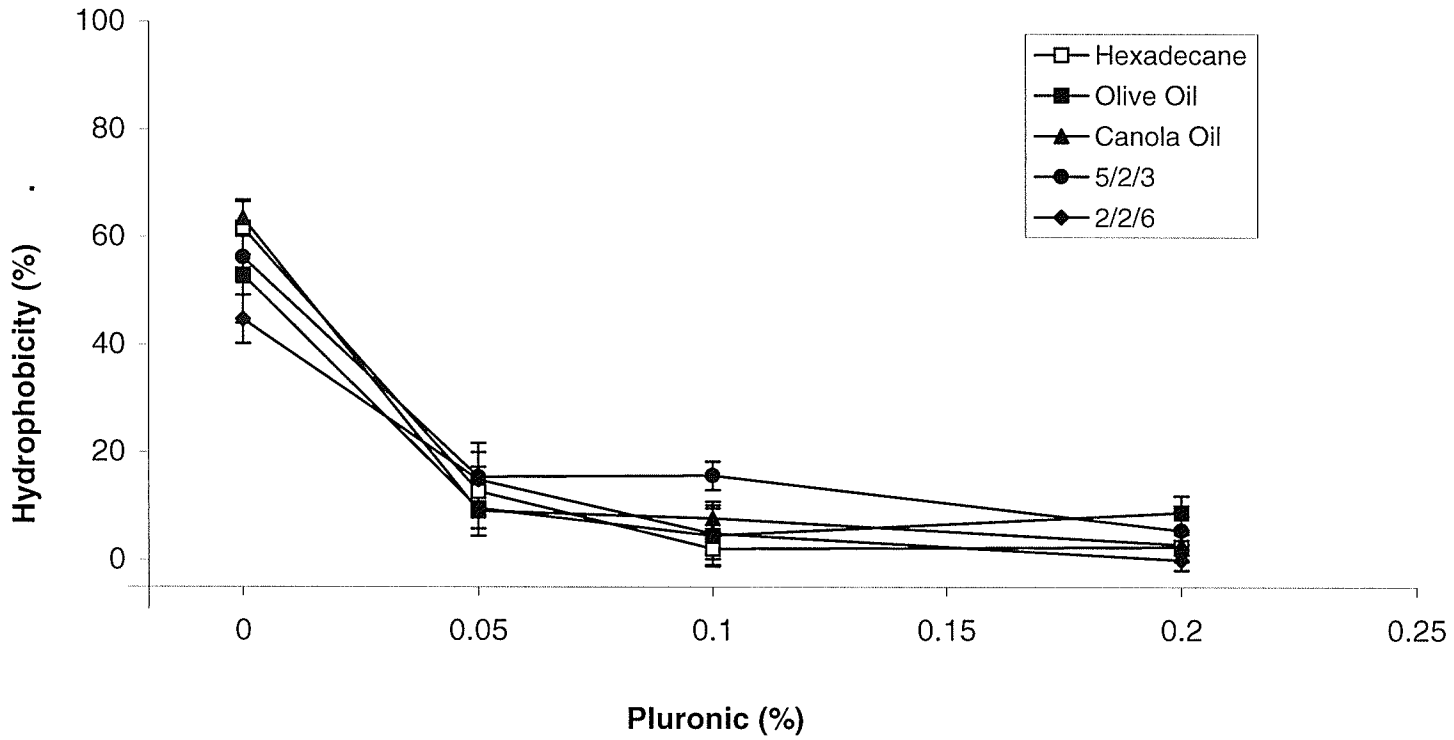


Figure 4.2 The effect of Pluronic F68 on the hydrophobicity index of CC9C10 hybridomas.

CC9C10 cells at a density of 10^6 cell/mL were taken from growth medium and re-suspended in PBS. The cell suspension was treated in the same way as described in Figure. 1. Determinations were repeated five times with different non-polar liquid phases – hexadecane, olive oil, canola oil and mixtures of the three at 5/2/3 and 2/2/6 (canola/hexadecane/olive v/v/v). Each point is a mean with an error bar (±SEM) where n=4.

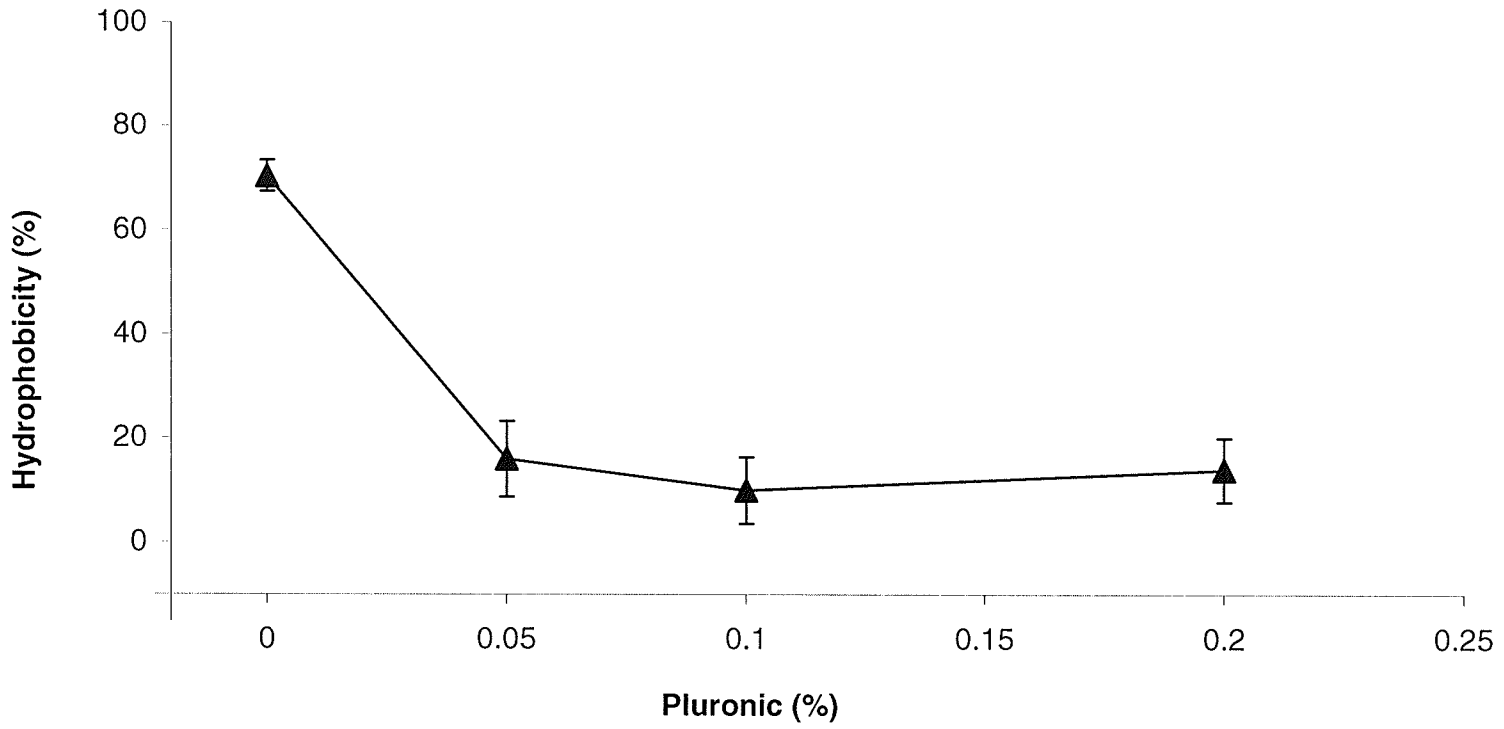


Figure 4.3 The effect of Pluronic F68 on the hydrophobicity index of SP2/0 myeloma cells.

Myeloma cells at a density of 10^6 cell/mL were taken from growth medium and re-suspended in PBS. The cell suspension was treated in the same way as described in Figure 1. Determinations were performed with canola oil as the non-polar liquid phase. Each point is a mean with an error bar (±SEM) where n=4.

4.0. The effect of bovine serum albumin (BSA)

Bovine serum albumin (BSA) is often added to medium as a protective agent in stirred cultures. Here the effect of BSA on the hydrophobicity index of the hybridomas, either on its own or in conjunction with Pluronic F68 was determined. Figure 4.4 shows that in the presence of 0.1 mg/mL BSA the hydrophobicity index of the cells decreased significantly from a value of 63 to 40. However, Pluronic F68 (0.05%) decreased the hydrophobicity index of the cells in both cultures to a value of zero. Thus BSA had no significant effect on the hydrophobicity indices of cells in cultures supplemented with Pluronic F68.

5.0 Effect of Pluronic F68 in agitated cultures:

In order to determine the effect of Pluronic F68 on cell viability in agitated cultures in the presence of bubbles, CHO 81 cells were introduced into four spinner flasks at a concentration of 10^6 cells/mL and then stirred at 500 rpm for 2.5 hours at different Pluronic F68 concentrations. The viable cells counts were taken every 30 minutes. Figure 4.5 shows the percentage of viable cell remained over the 2.5 hours period. Results show that Pluronic F68 protected the cells in a concentration dependent fashion. The maximum protection was offered with 0.2% Pluronic F68 where the viability of cells did not decrease over the testing period. A vortex occurs in the cultures at an agitation speed above 250 rpm. This leads to bubble entrapment and subsequent bubble bursting at the air-liquid interface.

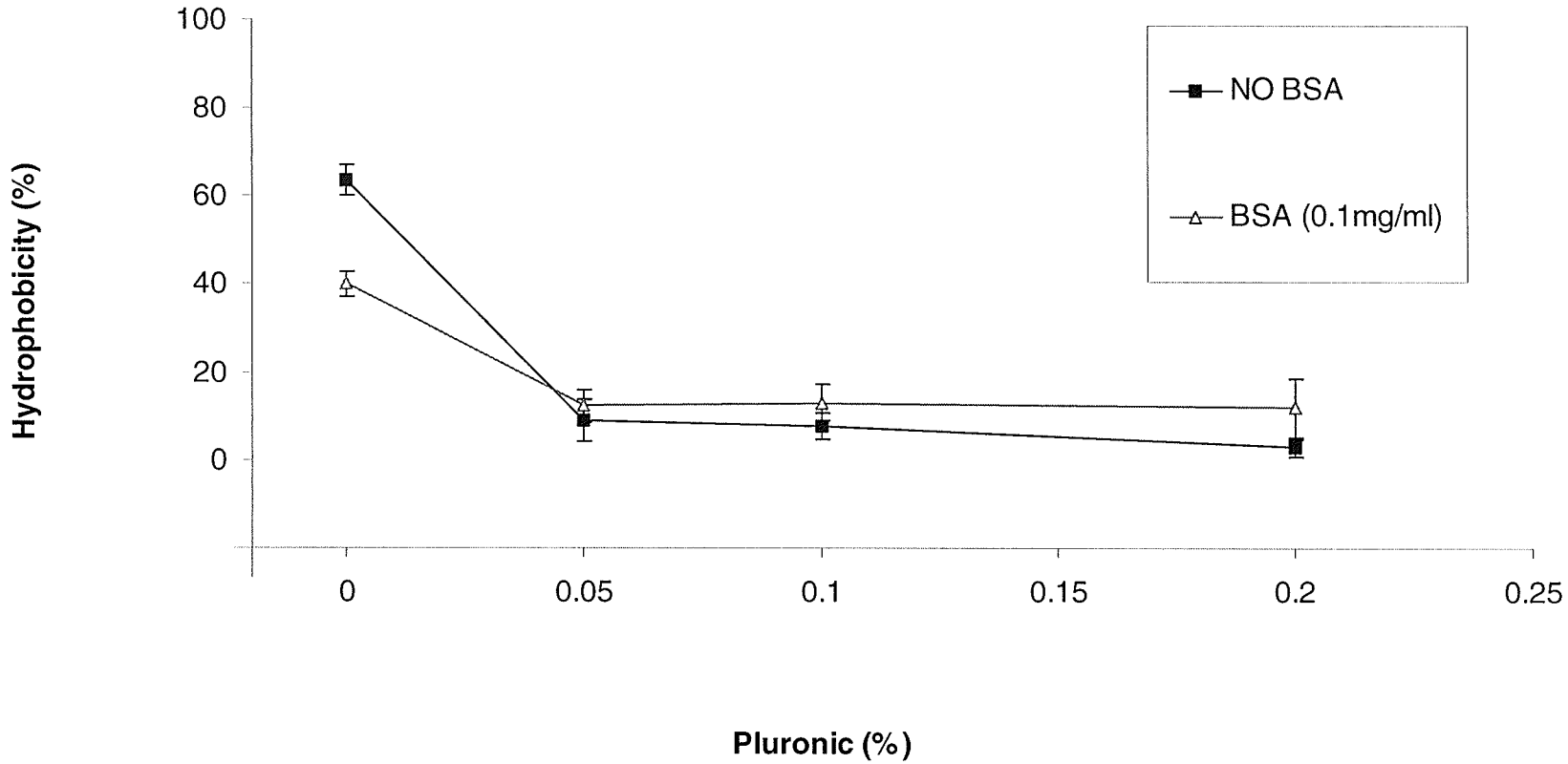


Figure 4.4 The effect of BSA on the hydrophobicity index of CC9C10 hybridomas.

CC9C10 cells at a density of 10^6 cell/mL were taken from growth medium and re-suspended in PBS with or without 0.1 mg/mL bovine serum albumin as indicated. Pluronic F68 was added to aliquots of these suspensions (2 mL) up to a concentration of 0.2%. The cell surface hydrophobicity index was determined for each sample using canola oil as the non-polar liquid phase. Each point is a mean with an error bar (±SEM) where n=4.

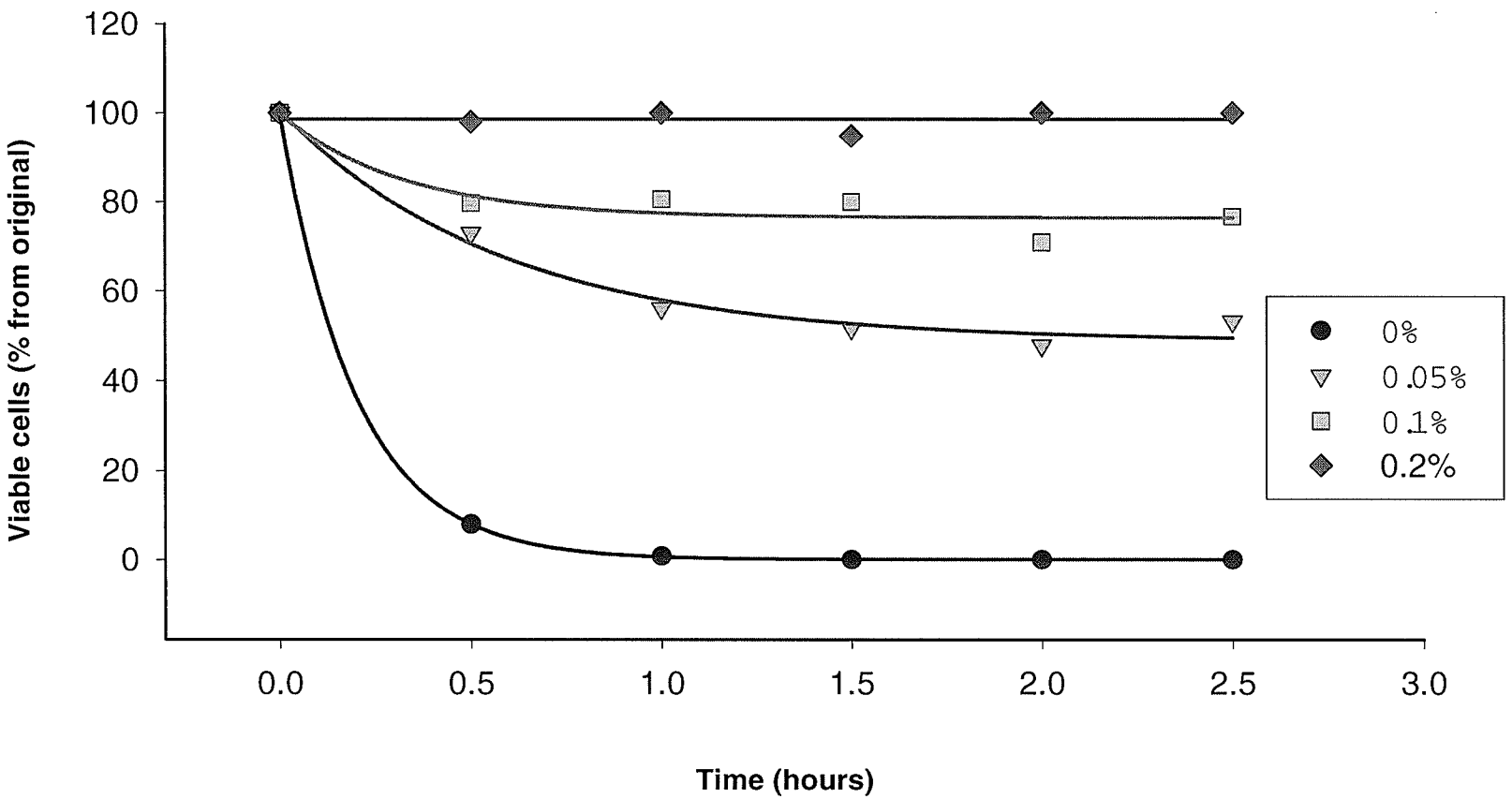


Figure 4.5 The Viability (Normalized) of CHO cells in spinner flasks agitated at 500 rpm at different concentrations of Pluronic F68.

Cells were tested in a density of 10^6 cells/mL in 100 mL spinner flasks and viable cells were counted at 30 min intervals. (N=1). Results are blotted as percent from the original count.

There is a correlation between the effect of Pluronic F68 on hydrophobicity index and its protective effect in spinner cultures. Hydrophobicity index of the CHO cells has decreased in a dose dependent manner and zero index was reached with a 0.2% concentration. Pluronic F68 also protected cells in spinners in a dose dependent manner and the maximum protection was noticed also at 0.2%.

Discussion:

Several methods have been reported in the literature for the measurement of cell-surface hydrophobicity (Magnusson and Johansson, 1977; Kjelleberg et al., 1980; Ener et al., 1992; Absolom et al., 1988; Rosenberg et al., 1980). However none of these have been applied to mammalian cells. In this report we have modified the BATH test based on adherence of cells to a hydrophobic phase to be suitable for mammalian cells (Rosenberg et al. 1980).

The hydrophobicity assay described in this report is based on the loss of cells from an aqueous suspension following vortexing with a hydrophobic oil phase. The cells are either removed into the oil phase or into the interface between the two solvent phases forming oil-cell droplets. The hydrophobicity index is a measure of the proportional loss of cells from the aqueous suspension. This index can be interpreted as a relative measure of the intrinsic cell surface hydrophobicity. Several parameters were shown to affect the results produced by the assay. A high initial cell concentration (10^6 cell/mL) reduced the error between replicates. Immediate vortexing of the 2-phase liquid mixture reduced cell aggregation and made more accurate determinations of the cell count.

Trypsinization of the CHO cells immediately prior to the assay resulted in a significantly higher hydrophobicity index.

The hydrophobicity index was determined for three cell lines: CHO, SP2/0 myeloma and CC9C10 hybridoma. The mean index of 82 determined for the CHO cells in the presence of canola oil was significantly higher than the indices of the other two cell lines (70 and 64 for the Sp2/0 and CC9C10 cells respectively). The similar values of the myeloma and the hybridoma is not surprising given that the Sp2/0 myeloma is the parental cell line of the CC9C10 hybridoma.

These differences in cell surface hydrophobicity might relate to the distribution of protein and carbohydrate molecules present on the outer cell surface and determine the adhesive properties of these cell lines. The CHO cells with the highest hydrophobicity index tend to form aggregates easily in suspension (Messi 1991; Renner et al. 1993) or grow attached to a solid substratum (Khang et al. 1995). On the other hand hybridomas and myelomas with significantly lower hydrophobicity indices have a lesser tendency to aggregate and normally grow as freely suspended cells in culture.

Several organic phases were tested in this assay with respect to three different cell lines and on the effect of Pluronic F68 on a range of concentrations up to 0.2%, which is in the range of Pluronic F68 concentrations commonly used in animal cell cultures (Butler 1990). Although all these non-polar organic solvents were suitable, canola oil and

hexadecane were considered to produce the most suitable results by maximizing the observable difference between Pluronic-treated and untreated cells.

Several mechanisms have been suggested for the protective effect of Pluronic F68 in agitated cell cultures. Pluronic F68 may change the physical properties of the gas-liquid interface by a decrease in the surface tension. This in turn could cause a decrease in the energy dissipated by bubble breakup where considerable cell damage has been reported in aerated cultures (Dey et al. 1997). Pluronic F68 may also protect cells against the potentially destructive effect of liquid shear forces in an agitated culture by strengthening the membrane (Zhang et al. 1992; chapter 5) or by plugging damaged portions of the membrane (Al-Rubeai et al. 1993).

An alternative and more important mechanism of protection offered by Pluronic F68 is by a reduction in the interaction between the cells and gas bubbles that arise from sparging in agitated cultures. Cell destruction in sparged, agitated cultures has been attributed to cell-laden bubbles being drawn into the liquid-gas interface where bubble disengagement occurs (Michaels et al. 1995). This zone is at the liquid-gas interface and is thought to be where the cells are subjected to forces that cause lysis (Kunas et al. 1990). A plausible explanation of the effect of Pluronic F68 in agitated cell cultures is that the polymer may interact with the cell membrane or the gas bubbles causing a reduction in the hydrophobicity of either and thus reducing the attachment of the cells to the bubbles.

Results suggest that the hydrophobicity assay described in this chapter provides a measure of the effect of Pluronic F68 on cells in culture. The most likely explanation is that Pluronic F68 causes a concentration-dependent reduction of the cell membrane surface hydrophobicity (CSH). The membrane of each cell contains a range of both hydrophobic and hydrophilic groups, the relative abundance of which gives each cell line unique physical properties. Pluronic F68 has a relatively high hydrophilic/ hydrophobic ratio, which could attach to the cell membrane causing an apparent reduction in overall hydrophobicity (Murhammer and Goochee, 1990). This explains the differences in effects between the cell lines that were tested.

An alternative explanation is that Pluronic F68 could modify the liquid interface and thus hinder the interaction of the cells with the oil phase. This is comparable with the idea that Pluronic F68 covers the air bubble surface in a sparged culture (Jordan et al., 1994) and has been suggested as the mechanism that Pluronic F68 protects proteins from denaturing at the air-liquid interfaces (Yuh-Fun et al. 1977). However, in this case the protective effect of Pluronic F68 requires only a significantly lower concentration (0.01%) to that used in our assay or in cell cultures.

BSA has also been reported to have a protective effect on cells in agitated cultures (Castro et al. 1996). Our assay showed that although BSA did decrease the hydrophobicity index, at the concentration normally used in culture this effect was not as great as that of Pluronic F68. The mechanism of action could be similar to that of Pluronic F68.

Our data show that Pluronic F68 has a significant cell line-dependent effect on the hydrophobicity index. The hydrophobic index of hybridoma and myeloma cells is reduced to a minimum at 0.05% Pluronic F68 whereas a significantly higher concentration of Pluronic F68 (0.2%) was required for a similar effect in CHO cells. The results suggest that these cell lines may be protected in agitated cultures at different concentrations of Pluronic.

When comparing the results of the hydrophobicity index with the protective effect of Pluronic F68 on the CHO in spinner cultures in the presence of bubbles we see a correlation with a dose dependent effect and complete protection at 0.2%. This correlation suggests that there may be a causal relationship between decreasing the hydrophobicity index of cells and a decrease in the cell to bubble attachment.

Pluronic F68 may have an adverse effect on product purification from cell cultures and so there may be an advantage in using the minimal concentration that offers cell protection in a cell culture bioprocess.

Conclusions:

- Pluronic F68 decreases the hydrophobicity index of CHO cells and CC9C10 hybridoma cells.
- The effect of Pluronic F68 on CHO cells is dose-dependent with maximum effect at 0.2% Pluronic F68.
- Pluronic F68 0.05 % is sufficient to produce a maximum decrease in the cell hydrophobicity of CC9C10 hybridoma cells.

- Pluronic F68 decreases the hydrophobicity index of SP2 myeloma in a similar pattern to its effect on hybridoma cells.
- Bovine Serum Albumin (BSA) decreases the hydrophobicity index of CC9C10 cells but not to the same extent as Pluronic F68.
- Pluronic F68 protects CHO cells in highly agitated cultures in a dose-dependent manner with maximum protection at 0.2% Pluronic F68.

Chapter 5 *: The protective effect of Pluronic F68 on mammalian cells in a bubble-free environment.

The main shear forces acting on cells in large-scale cell cultures are due to bubble bursting at the air-liquid interface. Bubbles in cell culture come from sparging which is used to increase the dissolved oxygen tension (DOT) in the culture medium. Another source of bubbles occurs in stirred cultures at high agitation speeds. Speeds above 250 rpm lead to gas entrainment due to the formation of a vortex.

The main protective mechanism of Pluronic F68 is dependent on ameliorating the damaging effects of bubble bursting at the top air-liquid interface (Zhang et al. 1992[b]; Michaels et al. 1995). However, there are mechanisms proposed for Pluronic F68 protection that may occur in the absence of bubbles. This protective effect of Pluronic F68 is dependent on increasing the cell resistance to shear stress. This chapter is devoted to studying the effect of Pluronic on the cell resistance to shear stress, in a bubble free environment.

1. The effect of Pluronic F68 on the growth of CHO 81 cells:

To study the effect of the Pluronic F68 in the absence of bubbles, a bubble free system is required. This system is available in small volume spinner flasks where agitation speeds lower than 250 rpm are usually sufficient and sparging can be avoided due to high surface area to volume ratio. In the absence of bubbles the shear force in a stirred culture

* The content of this chapter was submitted for publication.

system is derived from agitation. Another bubble free system is stationary cultures where no sparging or agitation occurs.

To study the bubble independent protective mechanism of Pluronic F68 CHO 81 cells were grown as stationary cultures in T-flasks as well as in spinner flasks at low agitation speeds (inoculation density of 10^5 cells/mL). The agitation speeds of the spinners were 45 rpm and 100 rpm where a vortex does not occur. In the spinner flasks the cells were cultured for 5 days and samples were taken daily. On the other hand cells were counted at the end of the T-flask 4 day cultures. Figure 5.1 shows the growth of CHO 81 cells in stationary cultures at different Pluronic F68 concentrations. Pluronic F68 (0.1%) increased the cell yield significantly ($P<0.001$) from $7.27 \pm 0.26 \times 10^5$ to $8.68 \pm 0.39 \times 10^5$ cells/mL (n=4) in stationary cultures. The growth stimulating effect was dose-dependent up to 0.1% Pluronic F68. The cells produced 348 ± 40 IU/mL (n=3) of EPO and the recombinant protein production was unaffected by the level of Pluronic F68.

Figures 5.2 shows the growth of CHO 81 cells at different Pluronic F68 concentrations in agitated spinner flasks at 45 rpm (n=5) and 100 rpm (n=4) respectively. The cells at the lower agitation speed of 45 rpm achieved significantly (ANOVA; $p<0.001$) higher cell densities compared with cells at higher agitation speed. On day 4 and in the absence of Pluronic F68, cells agitated at 45 rpm reached 7.57×10^5 cells/mL while cells agitated at 100 rpm reached only 6.02×10^5 cells/mL. There was no significant interaction between the Pluronic F68 concentration and the agitation rate and no significant effect of Pluronic F68 concentration on the cell concentration (ANOVA).

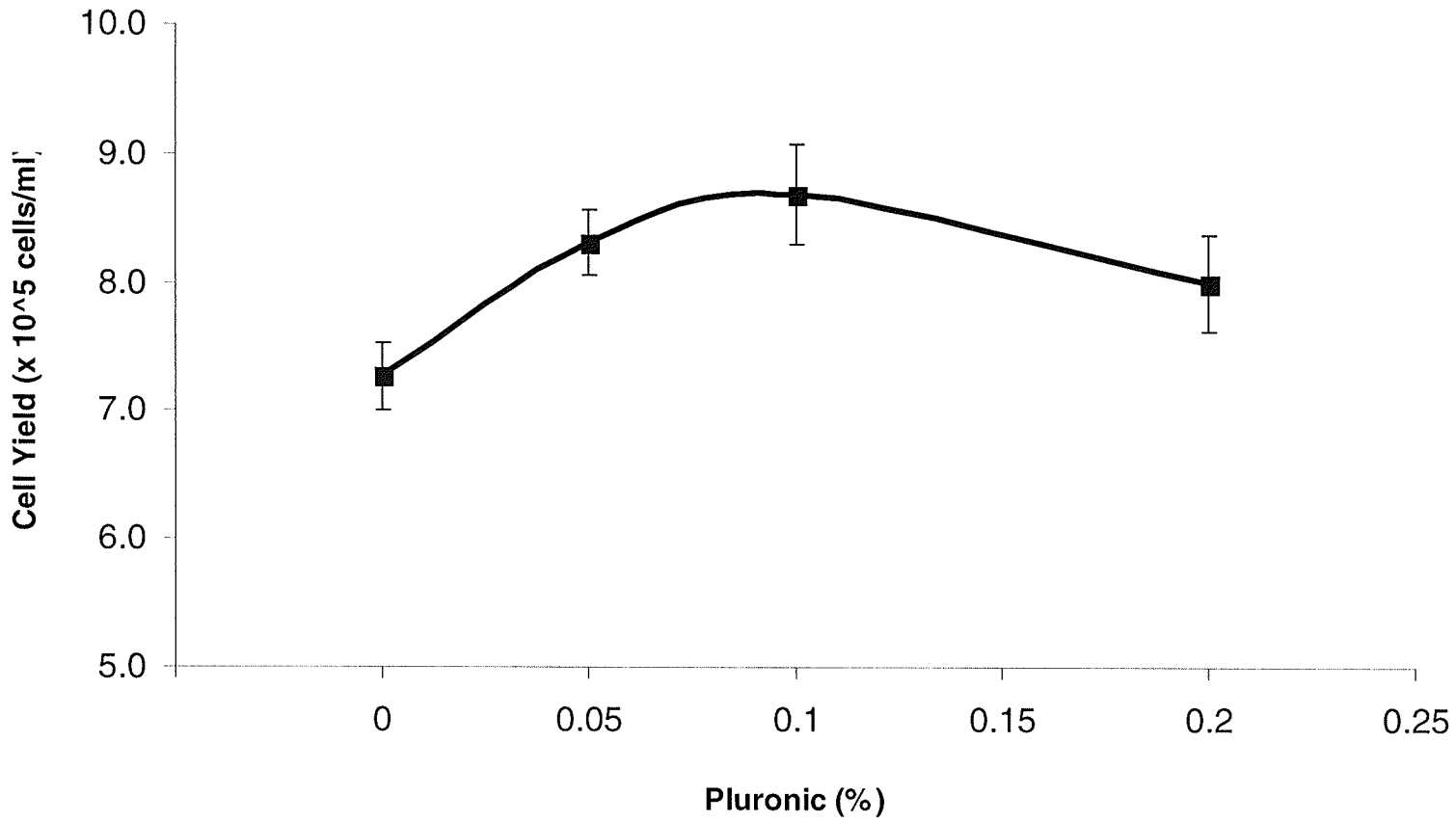


Figure 5.1 The growth of CHO 81 cells in stationary culture at different concentrations of Pluronic F68.

CHO 81 cells were cultured in 75 cm^2 T-flasks for 4 days with a seeding density of 10^5 cells/mL. Number of replicates (n) =4 consecutive passages. Error bars represent Standard Error of the Mean (SEM).

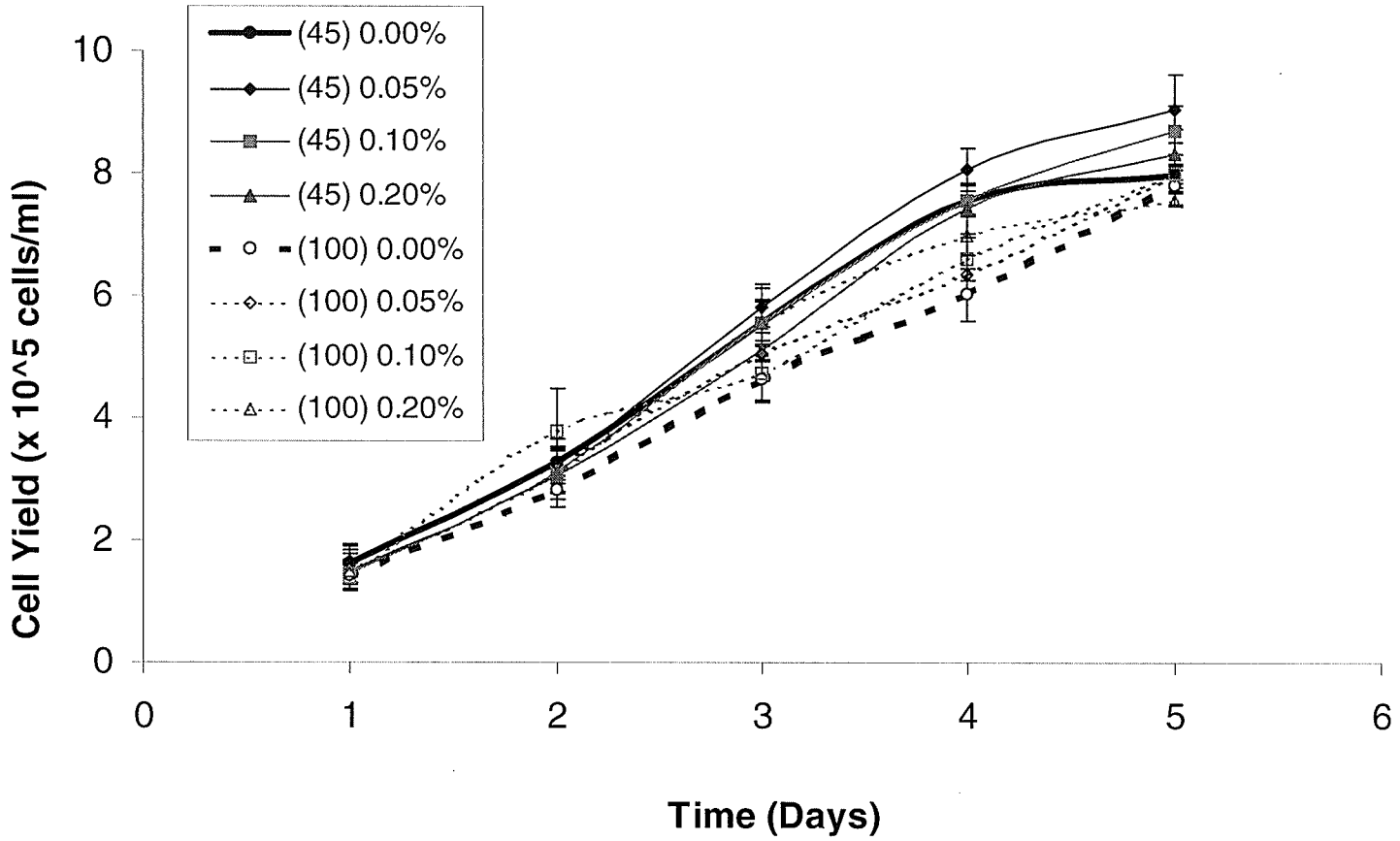


Figure 5.2 The growth of CHO 81 cells in stirred cultures at two agitation speeds of 45 and 100 rpm with different Pluronic F68 concentrations.

Cells were cultured in 100 mL spinner flasks samples were taken daily (n=5 and 4 for 45 and 100 rpm respectively) Error bars represent SEM.

The results were compared with those from a flask agitated at a high rate where bubble entrainment occurs. Figure 5.3 (duplicate of 4.5) shows the viable cells remaining after a high agitation rate of 500 rpm over a period of up to 2.5 hours. CHO 81 cells were introduced into four spinner flasks at a concentration of 10^6 cells/mL and then stirred at 500 rpm for 2.5 hours at different Pluronic concentrations. The viable cell counts were taken every 30 minutes.

Results show that Pluronic F68 protected the cells in a concentration dependent fashion. The percentage of viable cells remaining was significantly dependent on the concentration of Pluronic F-68 in the medium (n=1). In the absence of Pluronic F68 no viable cell remained while 100% of original viable cells remained after the 2.5 hour period of experiment in the presence of 0.2% Pluronic F68. Therefore it can be concluded that in the presence of bubbles Pluronic F68 showed a significant and protective effect in a dose-dependent manner. On the hand in the absence of bubbles Pluronic F68 showed no significant effect on cell concentration.

2. Choosing a viscometer to generate high shear forces in the absence of bubbles

Since stationary cultures and low agitation speed cultures produced low shear rates we proposed that the protective effect of Pluronic F68 in a bubble free environment might be evident only at higher shear forces. To examine this a viscometer that produces high shear rates in a bubble free environment was needed.

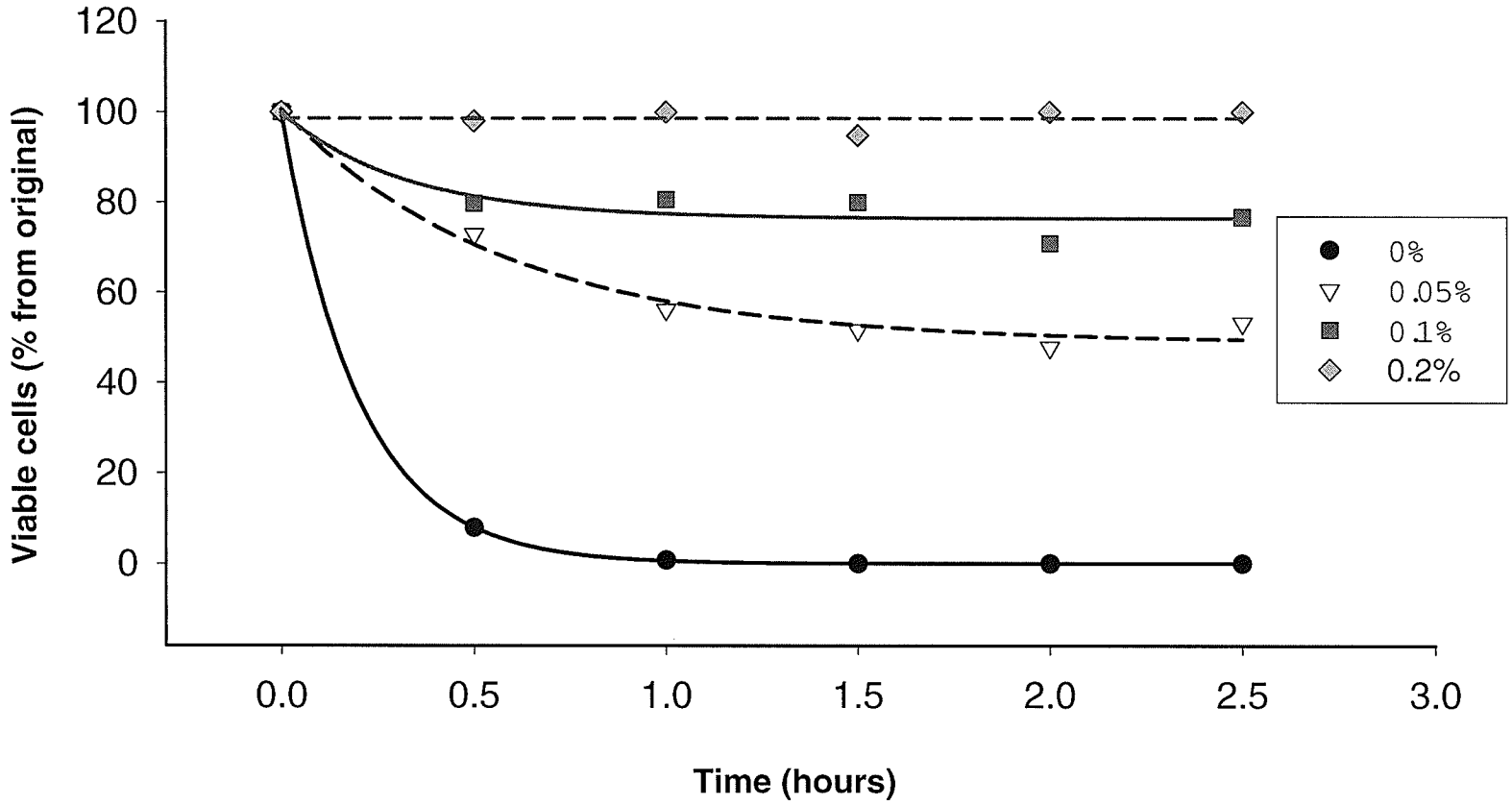


Figure 5.3 The viable cell concentration (Normalized) of CHO 81 cells in spinner flasks agitated at 500 rpm at different concentrations of Pluronic F68.

Cells were tested at a density of 10^6 cells/mL and viable cells were counted at 30 min intervals (n=1).

Several viscometers were tested including Rheomat 180, Brookfield CAP 2000 and Rheolab MC1. The Rheomat 180 viscometer is a spindle and cylinder viscometer that can produce a maximum shear rate of 3219 s^{-1}, which was not enough to test CHO 81 shear sensitivity. CHO 81 cells required 5 hours of shearing to completely damage all the cells (figure A.3 in Appendix). During this time the viability of the control sample of un-sheared cells decreased in PBS at room temperature to around 80 % possibly due to lack of nutrition (Figure A.1). The Brookfield CAP 2000 is a cone and plate viscometer that produces a shear rate of up to 26,666 s^{-1}. At this shear rate a complete decay curve was produced within 6 minutes of shearing giving the advantage of quick results. However, the sample volume was a maximum of 80 to 100 µl. This small sample volume increased the variability of results and the standard error of the mean (SEM) was up to 13% for some measurements (Figure A.4). Finally the Rheolab MC1 cone and plate viscometer was chosen because it produced shear rates of up to 12,000 s^{-1} enough to produce a complete decay curve within 10 min. The sample volume was up to 600 µl decreasing the variability of the measurements. Furthermore this viscometer had a controllable gap between the cone and plate permitting the choice of an optimum gap. Large gaps can encourage the entrainment of bubbles while smaller ones can lead to friction between the cone and the plate at certain points. The optimum gap size was found to be 0.025 mm. The temperature of the shearing plate was also controllable using circulating water (Figure A.5).

3. Choosing optimal testing conditions.

Temperature has been shown to affect the plasma membrane fluidity of cell membranes (Calder et al. 1994), which as a result might affect the shear sensitivity of cells. It was important to test the optimum temperature to be used for the shearing experiments. Figure 5.4 shows the effect of temperature on the sensitivity of CHO 81 cells to shear. Samples of cells (0.55 mL) suspended for at least 30 min at either 22° C or 37° C were subjected to a shear rate of 12,000 s^{-1} at the same corresponding temperature. The higher temperature increased significantly the shear sensitivity of CHO 81 cells. The viable cells concentration at 37 °C decreased to 13 % of the original value after 12 minutes of exposure to shear while at 22° C, 65% of viable cells remained over the same time period. Therefore temperature had significant effect on the shear sensitivity of the cells.

New batches of media were prepared for further experiments. The sensitivity of cells was consistently lower with freshly prepared medium compared with medium prepared >2 months previously. The difference may be due to deterioration of some medium components over time. Trypsinization of cells for 30 to 100 seconds altered significantly the cell resistance to shear. Using the CAP 2000 viscometer at 26,666 s^{-1}, 11% of viable cells trypsinized for 30 seconds remained after 4 minutes of shearing, while 66% of cells trypsinized for 100 seconds remained after the same shearing period (figure A.2 in the Appendix). Therefore in order to standardise all shear sensitivity measurements, cells were cultured in agitated culture to avoid the need for trypsinization, measurement were made at 37 °C, and a new fresh medium was used in all experiments.

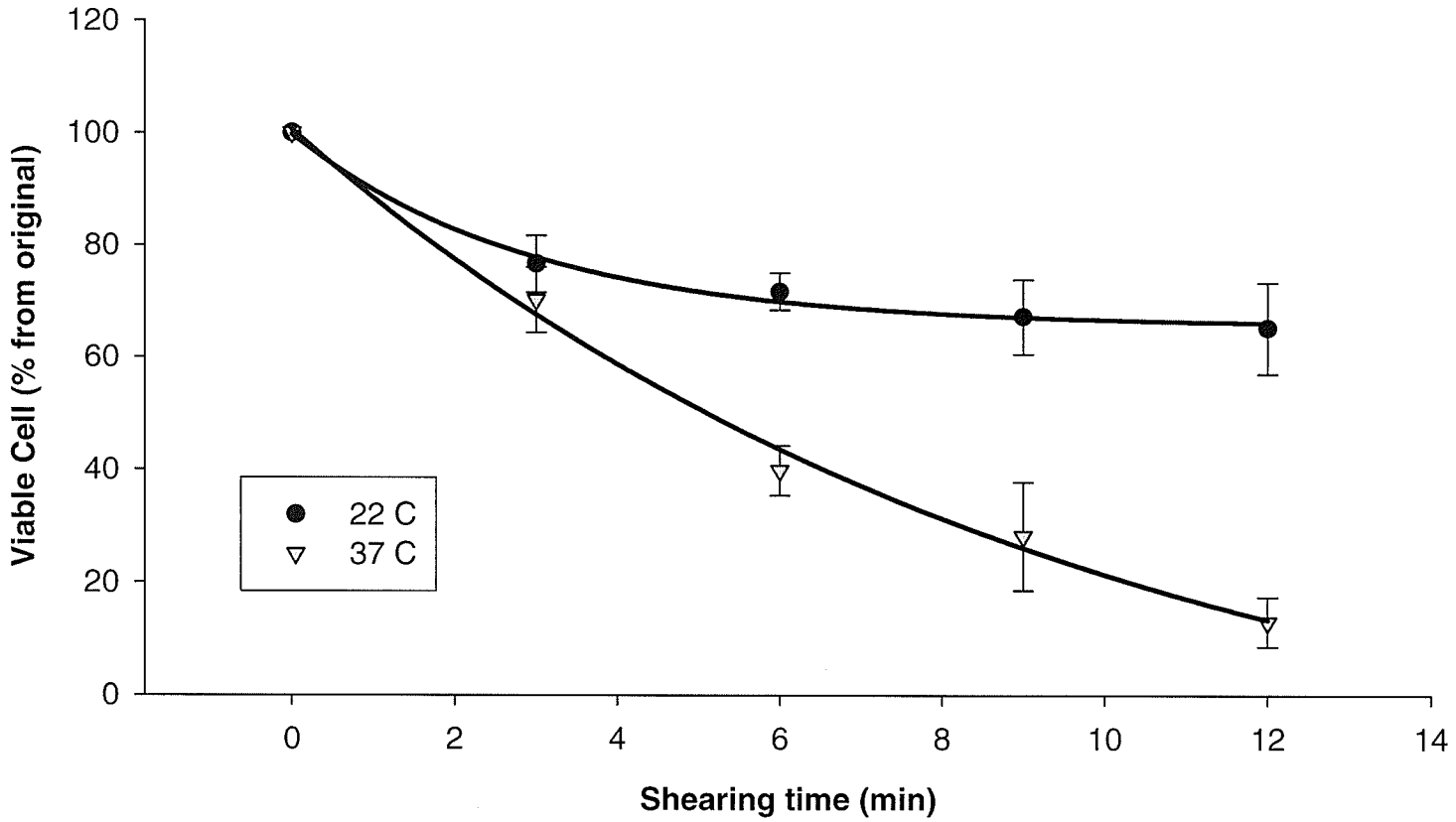


Figure 5.4 Effect of temperature on the shear sensitivity of CHO 81 cells under 12,000 s^{-1} shear rate.

Cells were kept suspended for at least 30 min at either 37 °C or 22 °C followed by shearing at 12,000 s^{-1} at the same temperature. Error bars represent standard error of the mean (n=4).

4. The effect of Pluronic F68 on mammalian cells in the viscometer:

Figure 5.5 shows the effect of Pluronic F68 on the CC9C10 cells (0.55 mL) after exposure to shear rates of 11,000 s^{-1} at a constant temperature of 37 °C. Prior to this experiment the cells were cultured in a Pluronic-free growth medium. The cells were then re-suspended in medium containing the Pluronic F68 concentrations indicated. The viable cells in the absence of Pluronic F68 decreased to 50% after 9 minutes of exposure to shear. However in the presence of 0.1 % Pluronic F68, 100% of viable cells remained over the same time period. Concentrations of 0.05% and 0.02% provided intermediate levels of protection. Therefore the protective effect of Pluronic F68 was dose dependant.

Figure 5.6 shows the effect of Pluronic F68 on CHO 81 cells during exposure to a shear force. The same experimental procedure was used as described in Figure 5.4. Prior to this experiment all the cells were cultured in a standard growth medium containing 0.1% Pluronic F68. The viable cell concentration in the absence of Pluronic F68 decreased to 9% after 9 minutes of shearing. However in the presence of Pluronic F68, 70% of viable cells remained at the same time period. The 0.1% Pluronic F68 concentration showed no further protection than 0.05% concentration.

To compare between the sensitivities of the two cell lines to shear stress, the decay curve of the cell lines in the absence of Pluronic F68 were re-plotted together. Figure 5.7 shows the difference in the shear sensitivity of two mammalian cell lines, CC9C10 hybridoma cells and Chinese Hamster Ovary (CHO) cells. The results show clearly that CC9C10 cells were significantly more shear tolerant than CHO 81 cells (T-test; $p < 0.001$). While 0

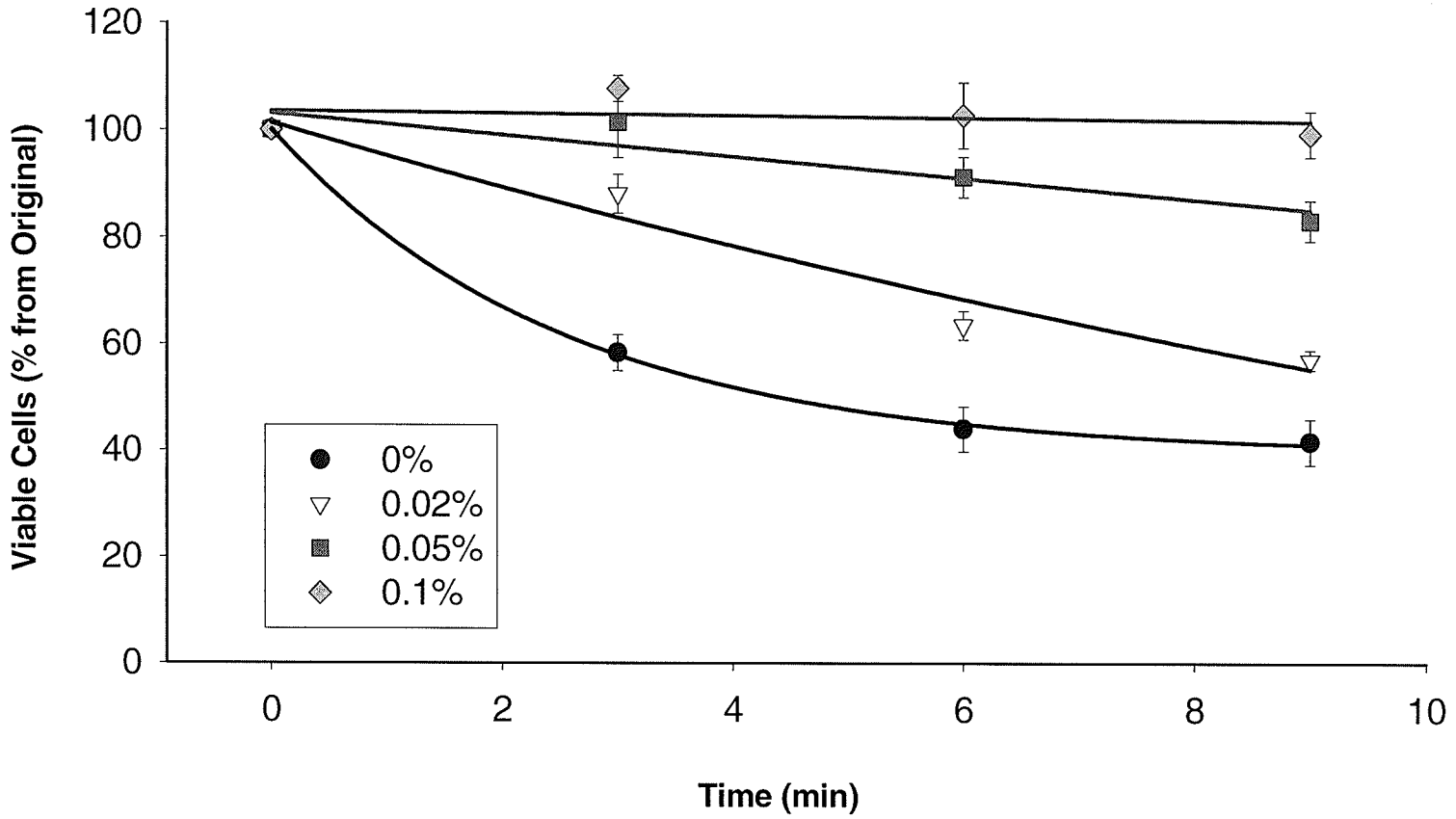


Figure 5.5. The effect of different concentrations of Pluronic F68 (%) on the shear sensitivity of CC9C10 cells.

A sample of cell suspension (0.55 mL) in a protein free medium were placed between a cone and a plate of Paar Physica viscometer. Cells were subjected to a shear rate of 11,000 s^{-1} at a temperature of 37 °C. Error bars represent standard error of the mean (n=4)

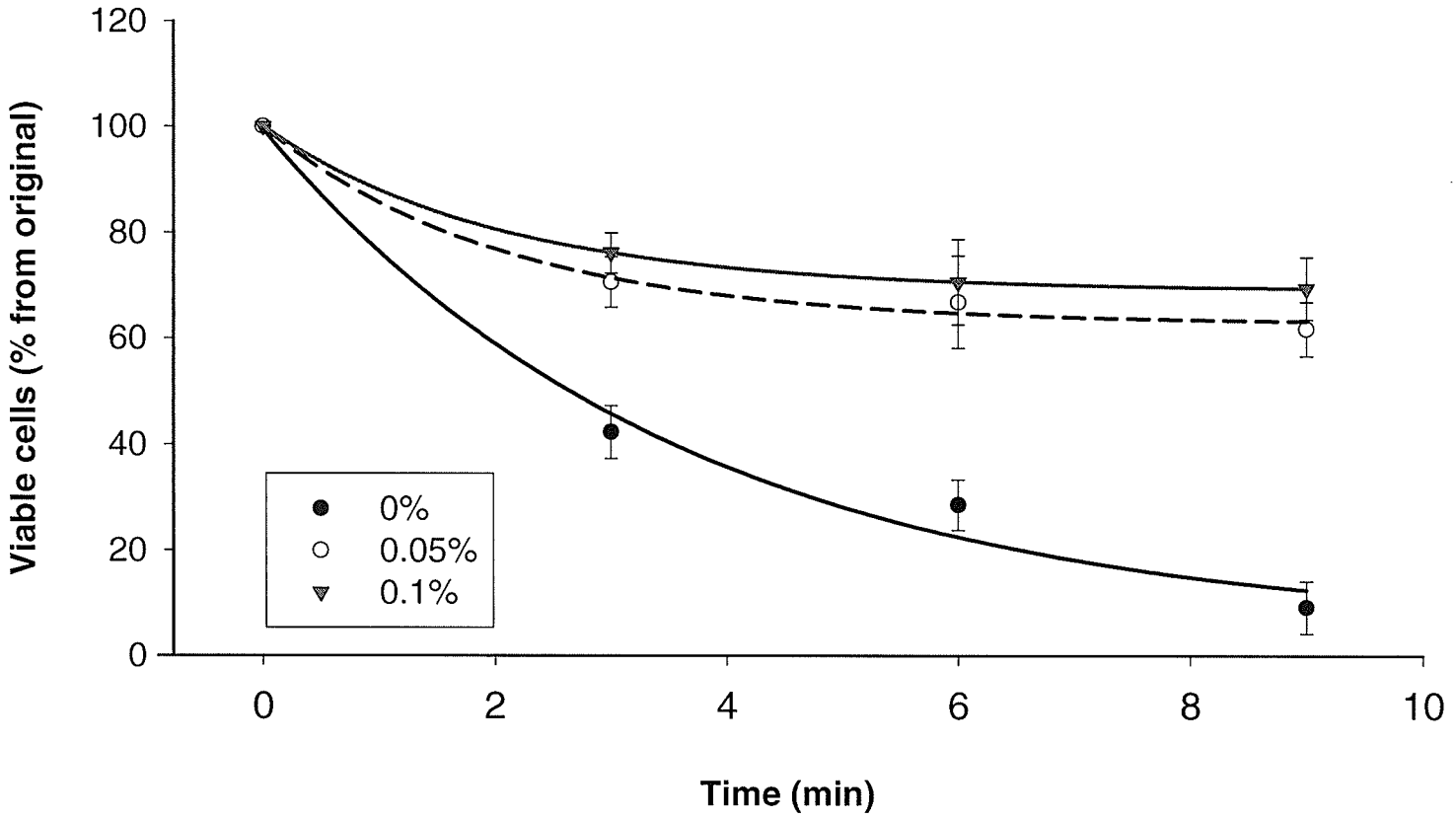


Figure 5.6 The effect of different concentrations of Pluronic F68 on the shear sensitivity of CHO 81 cells.

Cells were subjected to a shear rate of 11,000 s^{-1} at a temperature of 37 °C (as figure 5.5). Error bars represent standard error of the mean (n=4).

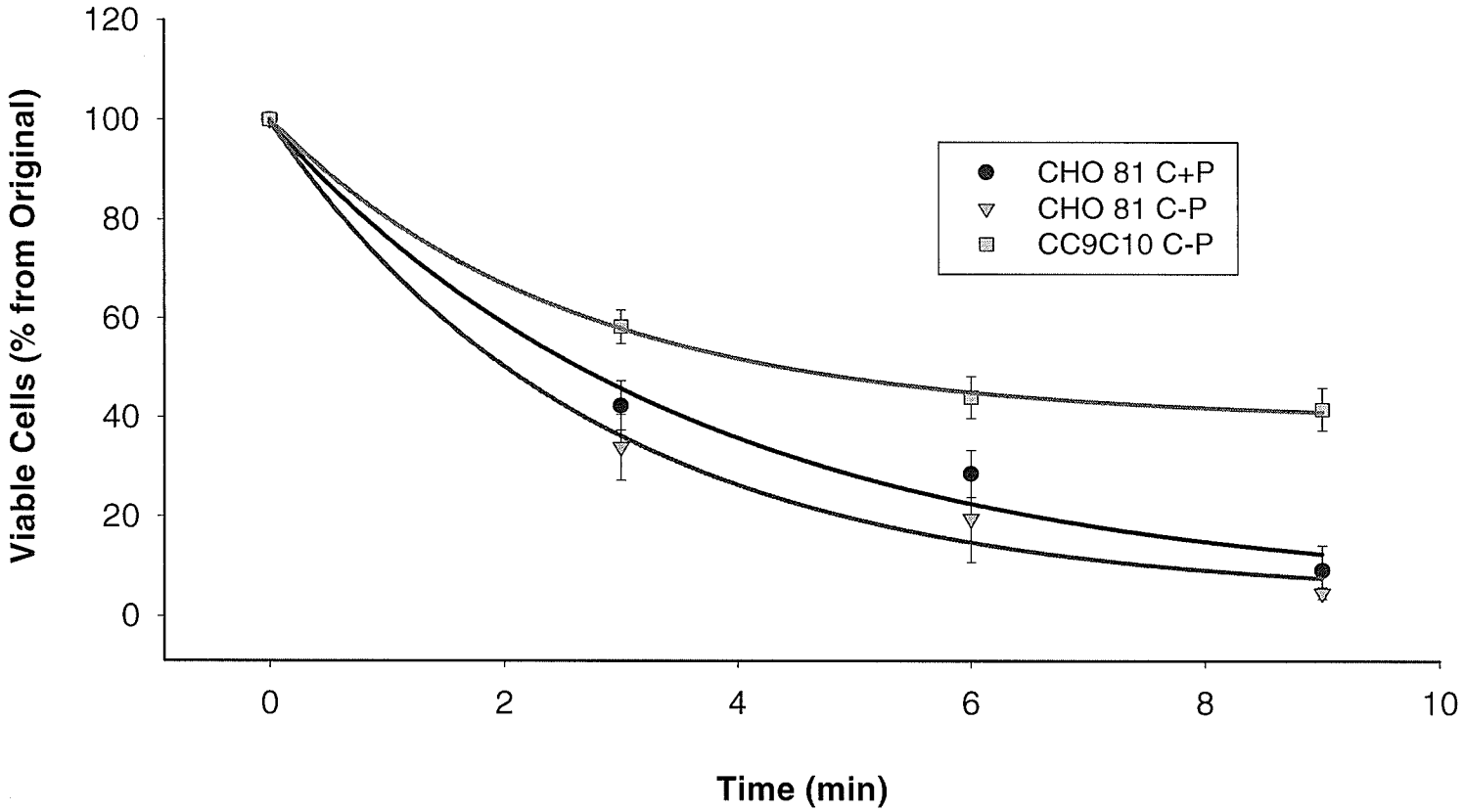


Figure 5.7 Comparison between the sensitivity of CC9C10 hybridoma cells and CHO 81 cells.

CHO 81 cells were cultured in the presence (C+P) and absence (C-P) of 0.1% Pluronic F68. CC9C10 cells were cultured in the absence of Pluronic F68. Conditions are the same as Figures 5.5 and 5.6. Cells were subjected to a shear rate of 11,000 s^{-1} and temperature of 37 °C. In all cases Pluronic F68 was absent during the shearing time experiment. Error bars represent standard error of the mean (n=4).

to 10% of viable CHO 81 cells remained after 9 minutes of shearing, 40% of CC9C10 cells remained after the same time period. The difference in sensitivity to shear might be due to several factors including the difference in average cell size and the strength of the membrane and the cytoskeleton of cells.

The standard CHO 81 cells growth medium contained 0.1% Pluronic F68 while the growth medium of CC9C10 hybridoma was Pluronic free. To exclude the possibility that the difference between the shear resistance of the two test cell lines was because of the presence of Pluronic F68 during the culture period an experiment was set up. The shear sensitivity of CHO 81 cells cultured in the presence or absence of Pluronic F68 was measured. The cells were then suspended in a Pluronic-free medium before exposure to shear in the viscometer. The results (Figure 5.7) showed no significant difference between the two conditions (T-test; $p = 0.09$). Therefore there was no significant effect of the presence of Pluronic F68 during cell growth on the sensitivity of the cells to the applied shear forces. We conclude that Pluronic F 68 had only a short-term effect while present in medium during the exposure to the shear force

5. Effect of Pluronic F68 on the viscosity of Growth Medium.

Compounds like dextran have been shown to increase the cells' resistance to shear by an increase of the medium viscosity. To investigate whether Pluronic protects the cell from shear forces by increasing the viscosity of the growth medium we measured the viscosity with increasing concentrations of Pluronic F68. The viscosity was measured using a Rheolab MC1 cone and plate viscometer at shear rates of 10,000 s^{-1}. The samples were

sheared for 2 minutes and the viscosity (Pa·s) was calculated by dividing the resulting shear stress (Pa) by the shear rate (s^{-1}) according to the equation $\tau = \beta \gamma$. Results are displayed as viscosity relative to water. Figure 5.8 shows the effect of Pluronic F68 on the viscosity of cell culture medium. The viscosity of the growth medium gradually increased slightly with higher Pluronic F68 concentrations. However even with the highest concentration in cell culture (i.e. 0.2%) the viscosity was very close to that of water. A concentration as high as 0.5% is rarely used in cell cultures. We conclude that the Pluronic F68 effect on viscosity is negligible. Therefore this cannot be a factor in any increase in the cell resistance to shear stress in a bubble free environment.

Discussion:

It has previously been shown that the major mechanism of the protective effect of Pluronic F68 is to decrease the cell- to-bubble attachment (Michaels et al. 1995, Zhang et al. 1992, Jordan et al. 1994). Pluronic F68 may also increase the cell membrane resistance to liquid shear. However, there are conflicting results in the literature. Zhang et al. (1992) have shown using micromanipulation techniques that the tension required to burst hybridoma cells increased significantly in the presence of Pluronic F68. Goldblum et al. (1990) reported the same effect on insect cells using a modified rheogoniometer (a device similar to a viscometer but with a rotating plate and a stationary cone) using higher concentrations of 0.2 and 0.3%. On the other hand, Michaels et al. (1991) reported no effect of 0.1% Pluronic F68 with hybridoma cells measured by a viscometer.

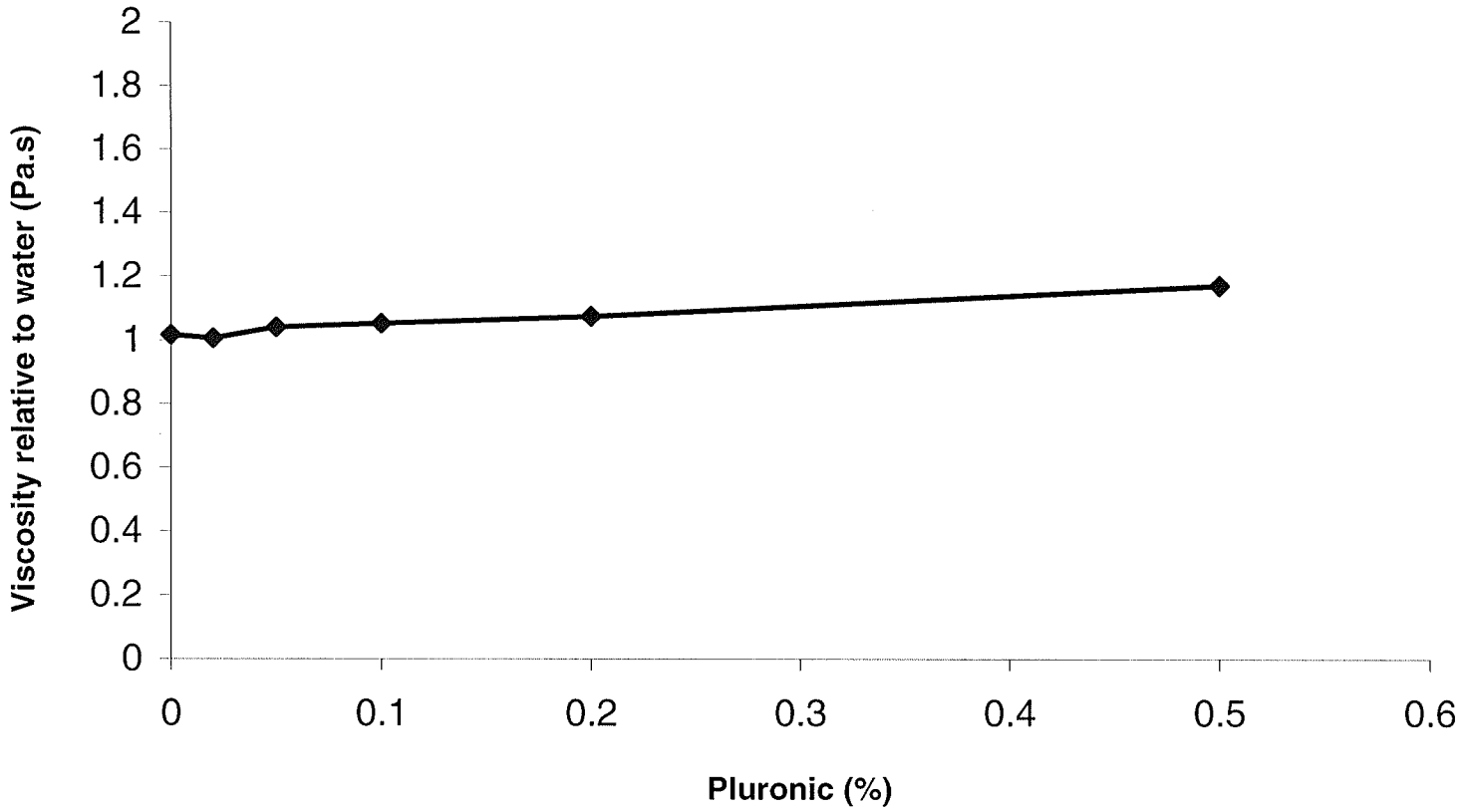


Figure 5.8 The effect of Pluronic F68 on the viscosity of growth medium (n=20).

The viscosity was measured using a cone and a plate viscometer at shear rates of 10,000 s^{-1} and sample were sheared for 2 minutes. The viscosity was calculated by dividing shear stress (Pa) by the shear rate (s^{-1}) according to the equation $\tau = \beta \gamma$.

In this chapter we have found that at low shear forces there was no significant effect of Pluronic F68 on CHO 81 cells as shown in the low agitated spinner cultures (45 and 100 rpm). At high agitation rates in spinner cultures (500 rpm) Pluronic F68 was significantly protective. However a high agitation rate (above 250 rpm) produces a vortexing effect in spinner flasks, which introduces bubbles into the medium. There are two potential mechanisms to cause cell damage: the bubbles entrained by the vortex and the stirring effect itself. Therefore a device that produces high shear forces but without bubbles was necessary to determine if Pluronic F68 could protect cells in liquid shear alone. The cone and plate viscometer produces shear rates of up to 12,000 s^{-1}.

The viscometer results presented here show that there was a significant protectant effect of Pluronic F68 on cells in a bubble-free environment. This agrees with the results of Zhang et al. (1992) who used a micro-manipulation technique and Goldblum et al. (1990) who used a modified rheogoniometer. Therefore the protective effect of Pluronic F68 in a bubble free environment was shown by more than two different techniques. On the other hand, Michaels et al. (1991), who also used a viscometer to test the sensitivity of hybridoma cells to high shear forces showed no significant effect of Pluronic F68 on the shear sensitivity of cells. However, Michaels et al. used a lower shear rate (5,000 s^{-1}) compared with the one described here (11,000 s^{-1}).

The possibility that Pluronic F68 interacts with cell membrane is supported by many reports in the literature. Ramirez and Mutharasan 1990 have shown that Pluronic F68 changes the fluidity of cell membrane. Wu et al. (1996 and 1997) reported that Pluronic

F68 can decrease the hydrophobicity of insect cell membranes. Results in chapter 4 also show that Pluronic F68 decreases the cell hydrophobicity index of CHO 81 and CC9C10 hybridoma cells. Decreasing the hydrophobicity index of cells suggest a mechanism of decreased cell-to-bubble attachment by Pluronic F68. Murhammer and Goochee (1990) have shown that the Hydrophilic Lipophilic Balance (HLB) of Pluronics is a very important criterion for predicting their ability to protect cells in culture, which suggests an interaction with the cell membrane. Furthermore, Wu et al. (1997) has measured the adsorption of Pluronic F68 molecules by measuring the decrease in the Pluronic F68 concentration in the growth medium. Al-Rubeai et al. (1993) also reported that Pluronic F68 could block a leaky cell membrane. Furthermore, Al-Rubeai et al. (1993) have found that Pluronic F68 protects surface associated immunoglobulin in mouse hybridoma (TB/C3) from shear damage and Lakhotia et al. (1993) found that Pluronic F68 can protect CD33 surface antigen of HL60 human leukemia cells. All these findings show that Pluronic F68 interacts with the cell membrane and therefore it may exert a protective effect on membrane resistance.

One possible mechanism by which Pluronic F68 might increase the cells' resistance to shear is by changing the plasma membrane fluidity of cell membranes. Change in temperature also can change the plasma membrane fluidity and thereby alter their sensitivity to shear stress. Higher temperature increases the PMF of cells by increasing the free rotations of atoms around carbon-carbon single bonds in long fatty acid chains. The effect of temperature on decreasing the sensitivity to shear is similar to the effect to Pluronic F68 suggesting the possibility of a common mechanism

The difference in the sensitivity of CHO 81 and hybridoma cells to shear can be due to intrinsic differences in the cell membrane or cytoskeleton, or due to a difference in the size of the cells. Larger cells are more prone to shear forces than smaller cells (Al-Rubeai et al. 1995). Under the microscope and using a haemocytometer CHO 81 cells are 1.5 times larger in diameter than hybridoma cells. When compared with the grid of the haemocytometer the approximate diameter of CHO 81 cells is in the range of 15 to 25 microns compared to CC9C10 hybridoma cells with a diameter range of 10 to 15 microns. Furthermore CHO 81 cells have been shown to have raffles sometimes depending on the method of culture compared with hybridoma cells with a smooth cell boundary.

The effect of Pluronic F68 in enhancing cell growth in stationary T-flasks has been reported previously by Keen and Rapson (1995). This could be due to the effect of Pluronic F68 on increasing the wetability of the culture substratum. An alternative mechanism could be an increase in the nutrient uptake as reported by Mizrahi (1995). Other authors explain this increase in growth by the impurities in commercial batches of Pluronic F68. However, the growth stimulatory effect of Pluronic F68 was apparent only in stationary cultures. It is possible that the interaction of Pluronic F68 with the substratum has an effect on its ability to stimulate growth of cells in stationary cultures. It has been shown also that Pluronic F68 competes with fibronectin for adsorption to surface flasks (Detrait 1999). There was no fibronectin in these experiments, nevertheless if Pluronic competes for the attachment to the flask surface then it could also interfere with the attachment of cells to the surface. This agrees with the findings of Bentley et al.

(1989) who reported a growth stimulatory effect of Pluronic F 68 while performing his experiments on attached cells. Bentley et al. did not report any effect of Pluronic F68 on suspension cultures. Another possibility is the presence of impurities affecting the attachment of cells to the flask surface and therefore promoting the cell growth.

Increasing the viscosity by compounds like dextran has been reported by Lakhotia et al. (1992) and Goldblum et al. (1990) to increase the cells' resistance to shear. Here we exclude this mechanism of action because Pluronic F68 did not increase significantly the viscosity of the medium at concentrations usually used in cell culture (up to 0.2%).

The sensitivity of cells to shear forces has an important implication on the design and culture parameters of bioreactors in large-scale cultures. This includes also the design of the impeller, the agitation speed and the intensity of sparging. The work presented here suggests that Pluronic F68 concentration and its use can be "tailored" to the cell type, the method of culture and bioreactor geometry. This is important because although Pluronic F68 is protective for cells in culture, it inhibits the growth of some cells in culture as shown at the 0.05% Pluronic F68 concentration by Rubeai et al. (1992). Pluronic F68 also might make the purification of some recombinant proteins more difficult especially if the molecular weight of the recombinant protein is not large enough and a 10 kDa, or less, filter is used for concentrating the supernatant (Pluronic F68 molecular weight is 8.4 kDa). This can be more important in protein-free media where Pluronic F68 might be the largest molecule among all the additives.

Conclusions:

1. Pluronic F68 stimulates the growth of CHO 81 cells in stationary cultures.
2. The protective effect of Pluronic F68 is not significant at low agitation speeds (45 and 100 rpm) with low shear rates.
3. Pluronic F68 protects cells in highly agitated cultures (500 rpm) in a dose-dependent manner
4. Pluronic F68 protects CC9C10 and CHO 81 cells at high shear rates in the absence of bubbles.
5. The protective effect of Pluronic F68 on CC9C10 cells (at high shear rate in the absence of bubbles) is dose dependent with full protection at 0.1% Pluronic F68.
6. The protective effect of Pluronic F68 (at high shear rate) is observed only while present in the culture medium. No significant residual effect was detected if Pluronic F68 was present only during the culturing period while absent during the shear testing.
7. The effect of Pluronic F68 on the cell culture viscosity is negligible and cannot be responsible for its protective effect.
8. A higher temperature increases significantly the sensitivity of cells to shear stress.

Chapter 6 ♣: Development of an Assay for the surfactant Pluronic F68 in Mammalian Cell Culture Medium.

The study of the interaction of Pluronic F68 and cultured cells is limited by the lack of a simple and sensitive assay for Pluronic F68. The purpose of the work described here was to develop such an assay. This assay was utilized later to measure the concentration of Pluronic F68 during cell culture as will be discussed later in chapter 8.

Three methods have been described in the literature for measuring Pluronics, the cobalt thiocyanate method (Boyar et al. 1977; Greff et al. 1965; Tercyak et al. 1990) the Wichbold method (Wichbold et al. 1972), and the potassium tetrakis(4-halophenyl) borate method (Tsubouchi et al. 1985). These methods were each originally developed for the analysis of non-ionic surfactants in river and sewage water. The Wickbold method (Wichbold et al. 1972) involved complex formation between Pluronic and tetraiodobismuthate. The precipitate was dissolved in ammonium tartarate and titrated with pyrrolidone dithiocarbamate. The method as described by Wickbold involved the use of a specifically designed apparatus for concentrating the complex. The other method developed by Tsubouchi et al. (1985) involved the titration of Pluronic with tetrakis(4-halophenyl)borate. In this method, Pluronic was extracted into the organic phase of a two-phase mixture, and the end-point was determined by detection of excess tetrakis(4-halophenyl)borate by an indicator, Victoria blue. In an attempt to develop a simple and

♣The content of this chapter was published in Ghebeh, H., Handa-Corrigan, A., Butler, M. (1998) Development of an assay for the measurement of the surfactant Pluronic F-68 in mammalian cell culture medium. *Analytical Biochemistry* **262**: 39-44

routine assay for Pluronic these two methods were dismissed as being too time consuming and requiring too large a sample volume.

A more promising method was originally developed by Greff et al. (1965) and later modified by Tercyak & Felker (1990). This involved the complexation of Pluronic with cobalt thiocyanate. The absorbance of the solubilised complex was measured spectrophotometrically and used as a quantitative measure of Pluronic F-68. The method was used for Pluronic F-68 determination in liver extracts present in the concentration range of 0.08-0.8% (w/v). In this work several modifications were made to the cobalt thiocyanate method in order to improve the sensitivity and reproducibility of this assay for measurement of Pluronic F-68 in mammalian cell culture medium.

3.1 Development of the assay:

The assay depends on the formation of a colored complex between Pluronic F68 and cobalt thiocyanate. The complex forms a precipitate that sediments upon centrifugation following the reaction of cobalt thiocyanate with Pluronic F-68. The precipitate dissolves in acetone and the intensity of color is proportional to the amount of Pluronic F68 present in the sample. The structure of the cobalt thiocyanate-Pluronic complex is postulated to be due to hydrogen, ammonium or cobalt ion (M^+) complexation with oxygen groups in ether to form *oxonium* ion which react with a suitable anion (X^-) like thiocyanate (Greff et al. 1965). The postulated structure of the complex is shown below (Greff et al. 1965):

$$[RO\underset{\overset{|}{M}}{CH_2}\text{-}(CH_2O\underset{\overset{|}{CH_3}}{CH_2})\text{-}(CH_2OCH_2)\text{-}CH_2OH\ ]^+ .\ X^-$$

However, it was found later that many oxygen atoms in the ether interact with the inorganic ion (heavy metal, alkali, or earth alkali ions) and that the complex forms a helix configuration to accommodate the ion in the center (Cross 1987). The color of the complex is due to the Pluronic-thiocyanate complex that has an absorbance spectrum with two peaks, one with a sharp absorbance peak at 328 nm and a second broader peak at 624 nm. The peak ratio for this complex is $A_{328}:A_{624}=4.4$ (Tercyak et al. 1990).

The results of the original cobalt thiocyanate method, as described by Tercyak et al. (1990), are reported in Figure 6.1(A). This method involved cutting the bottom of each test tube to eliminate the non-specific absorbance arising from surplus dye adsorbed onto the inner wall of the test tube. This step was found to be both impractical and hazardous. Therefore this was substituted with an alternative step in which the precipitate was washed with ethyl acetate. Washing with ethyl acetate reduced the background non-specific absorption and stabilized the Pluronic-thiocyanate complex. Figure 6.1(A) shows the absorbance of the complex at 624 nm relative to the concentration of Pluronic F-68 standards in distilled water. A linear relationship with a regression coefficient of 1.694 and a coefficient of determination (R^2) of 0.9862 was obtained. The sensitivity of this assay was similar to that reported by Tercyak et al. (1990). The lower level of detection of Pluronic F68 was 0.08%. Although this was the basis of a quantitative assay, the variability was too high and the sensitivity insufficient for use at the concentration

range normally found in cell culture medium (0.01-0.2%).

Several modifications were made to improve the sensitivity of the assay to enable measurement of Pluronic F68 concentrations at those commonly found in culture medium. The procedure was repeated using an increased sample volume (200 µl) and absorbance measurement at a wavelength of 328 nm for which a higher extinction coefficient has been reported for the complex (Tercyak et al. 1990). With these modifications, a linear relationship between the absorbance and the concentration of Pluronic F68 was achieved between 0.01% and 0.16% Pluronic F68 in distilled water (Figure 6.1 (B)). The regression coefficient was 12.88 and the coefficient of determination (R^2) was 0.9985. At concentrations of Pluronic F68 lower than 0.01%, precipitation of the complex was not apparent. An attempt to increase the sensitivity further by an increase in the sample volume to 300 µl was unsuccessful.

3.2 Application of the assay to culture media:

An initial attempt to adapt this assay to the measurement of Pluronic F68 in a serum-free cell culture medium (NB-SFM) resulted in the data shown in Figure 6.2 (A). The measured absorbance of the cobalt thiocyanate-Pluronic complex increased linearly with Pluronic F68 concentration up to a value of around 0.08%. However, an absorbance plateau was observed at around 1.5 AU for higher concentrations of Pluronic F68. This may be due to interference from other medium components resulting in the precipitation of non-specific complexes on the walls and the bottom of the tube.

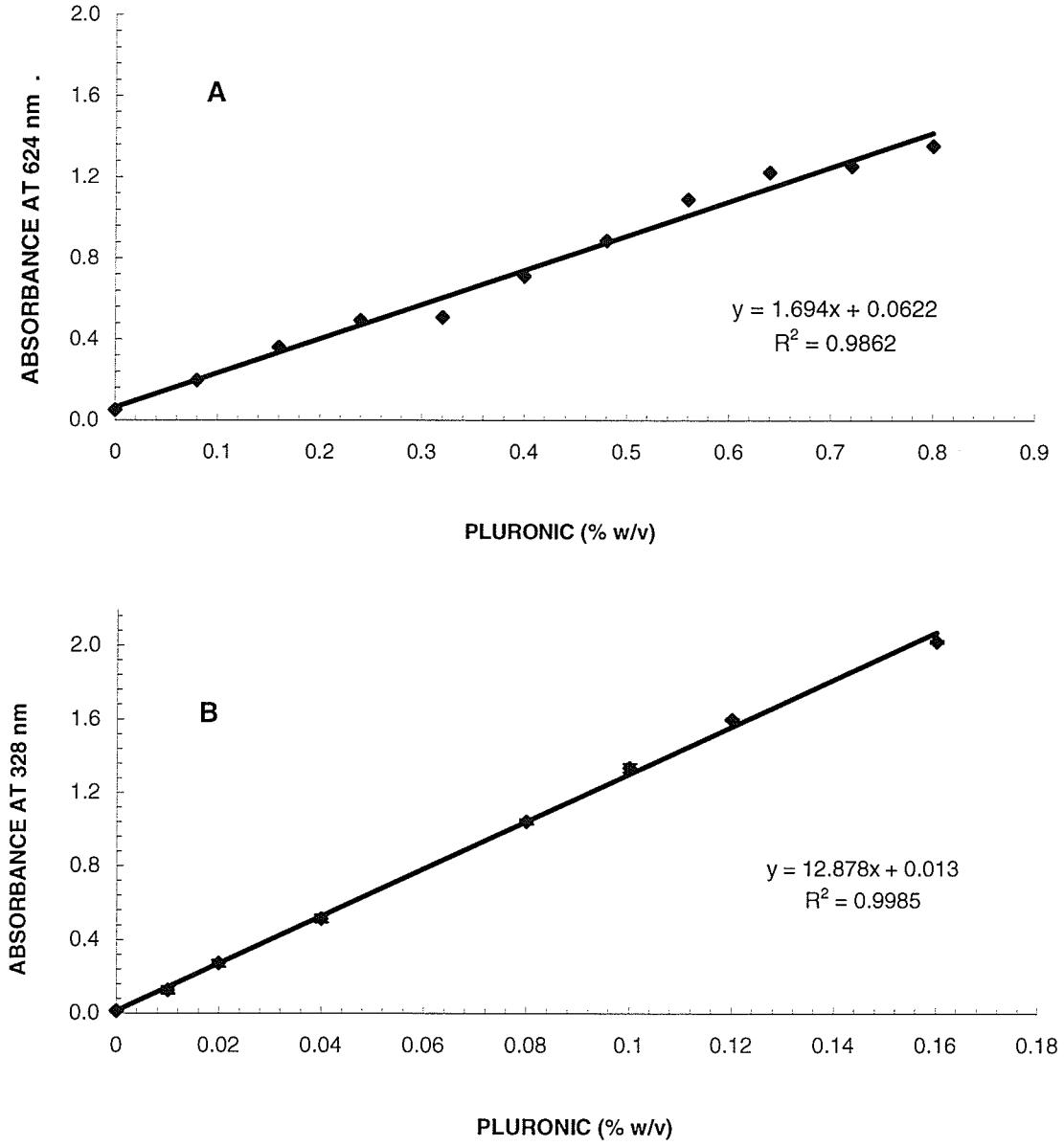


Figure 6.1 (A) Standard curve for Pluronic F-68 assay in distilled water (n=1).

The assay was modified from Tercyak & Felker (10). Pluronic F68 (100 µl) was added to a 1.5 mL microcentrifuge tube at a concentration ranging of 0.01 to 0.2%. This solution was mixed with 100 µl of the cobalt thiocyanate reagent and 200 µl ethyl acetate. After centrifugation for 1 min at 10,400 g, the two liquid layers were aspirated and discarded. The sediment was washed with 200 µl ethyl acetate and dissolved in 2 mL acetone. The absorbance was measured with a spectrophotometer at 624 nm.

(B) Improved standard curve for Pluronic F-68 assay in distilled water (n=3, ±SD).

The volume of each Pluronic F68 standard added was increased to 200 µl and the final dissolved precipitate was measured at 328 nm.

In an attempt to reduce this interference, ethanol (80 µl) was added to the initial assay mix. This resulted in reduced variability and a linearity of measurement from 0.01% to 0.2% Pluronic F68 as shown in Figure 6.2 (B). The regression coefficient was 9.28 and the coefficient of determination (R^2) was 0.9988. The volume of ethanol addition was critical since lower amounts than 80 µl could not dissolve all the non-specific precipitate, while higher amounts reduced Pluronic complex formation. Similar results were obtained for the measurement of Pluronic F68 in another serum-free medium, CNJsfm 2.1 (previously called APO-SFM), which is used routinely in our laboratory for CHO cell growth. In this case a regression coefficient of 10.00 and a coefficient of determination (R^2) of 0.9964 was measured over the same concentration range (Figure 6.3).

Application of this assay to a serum-based medium resulted in linearity and a sensitivity of measurement over a reduced concentration range of 0.04% to 0.16% Pluronic F-68 (Figure 6.4). Over this concentration range the regression coefficient was similar to that measured in serum-free medium at 9.894 and R^2 of 0.9992. It is likely that the higher protein content of the serum-based medium causes interference in Pluronic complex formation or its extraction at Pluronic F68 concentrations outside this range.

The assay described in this chapter provides a useful tool to study Pluronic F68 concentrations during cell culture. Such measurements are likely to be important during serum-free growth of cells in bioreactors where the cultures are often supplemented with Pluronic F68 to reduce potential cell damage from the hydrodynamic forces produced by

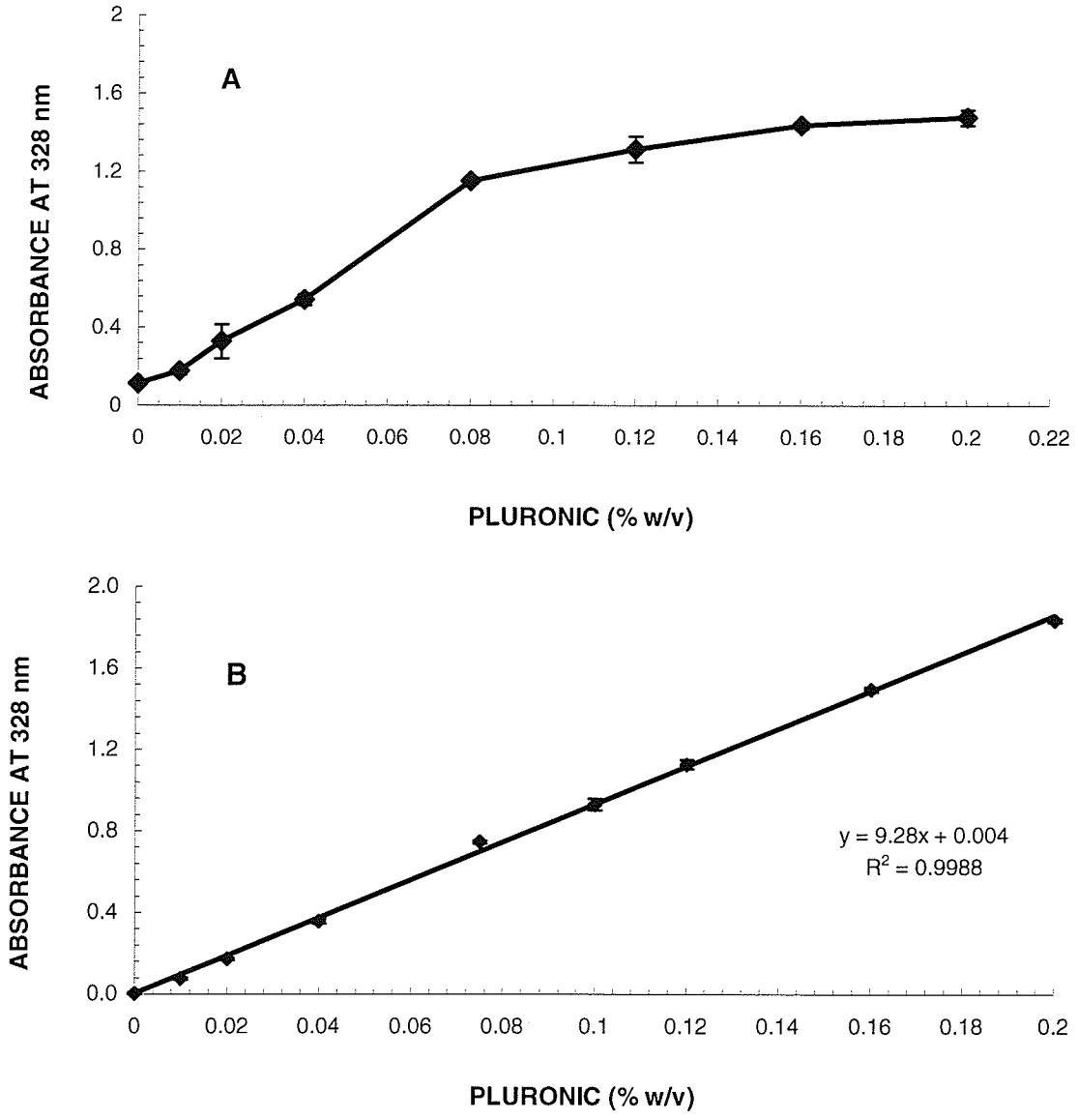


Figure 6.2 (A) Assay for Pluronic F-68 in serum-free culture medium (n=2, ±SD).
The protocol described in Fig. 1(B) was followed for standard Pluronic F68 samples dissolved in the serum-free medium, NB-SFM.

(B) Improved assay for Pluronic F-68 in serum-free culture medium (n=3, ±SD).
The protocol was modified by the addition of 80 µl ethanol.

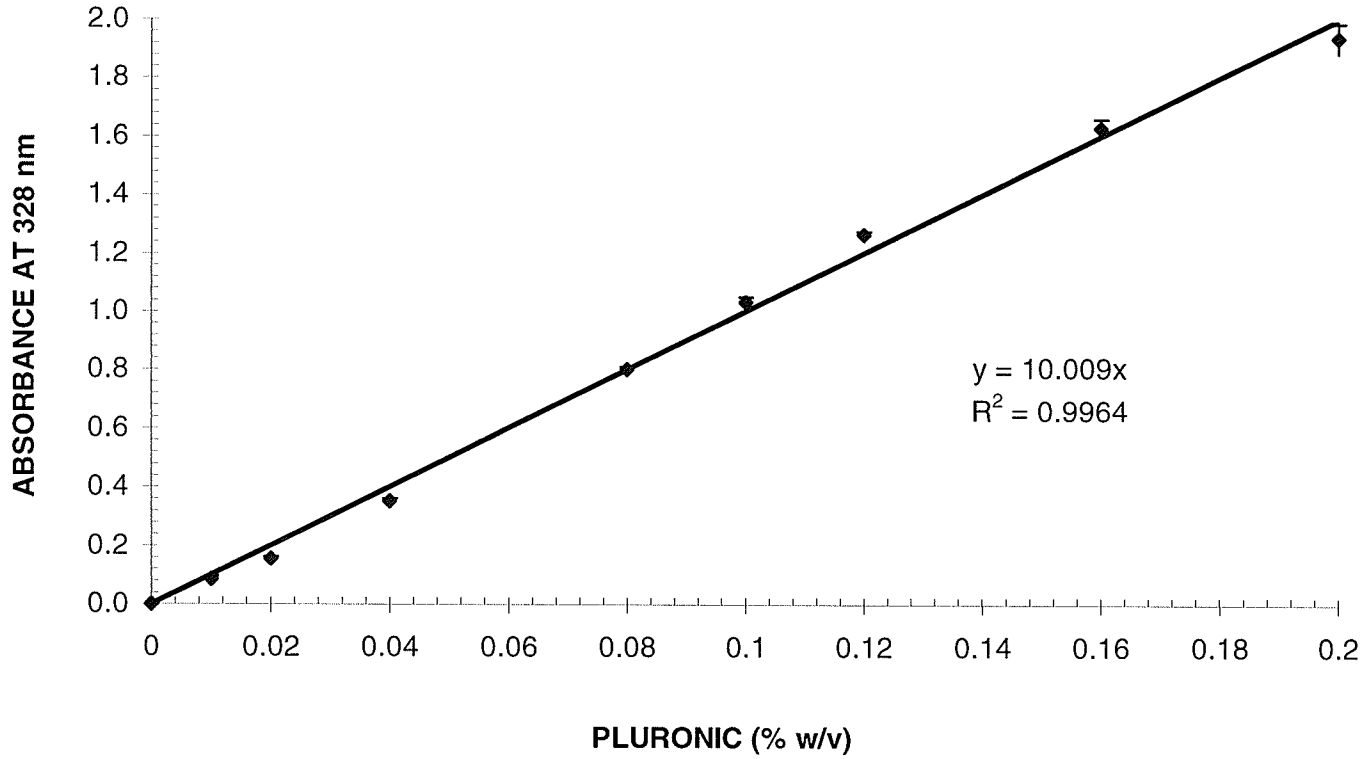


Figure 6.3 Standard curve for Pluronic F-68 in CNJsfm 2.1 medium (n=3, ±SD).

The protocol described in Fig. 2 (B) was followed for Pluronic F68 standards within the concentration range of 0.01-0.2% in CNJsfm 2.1 medium.

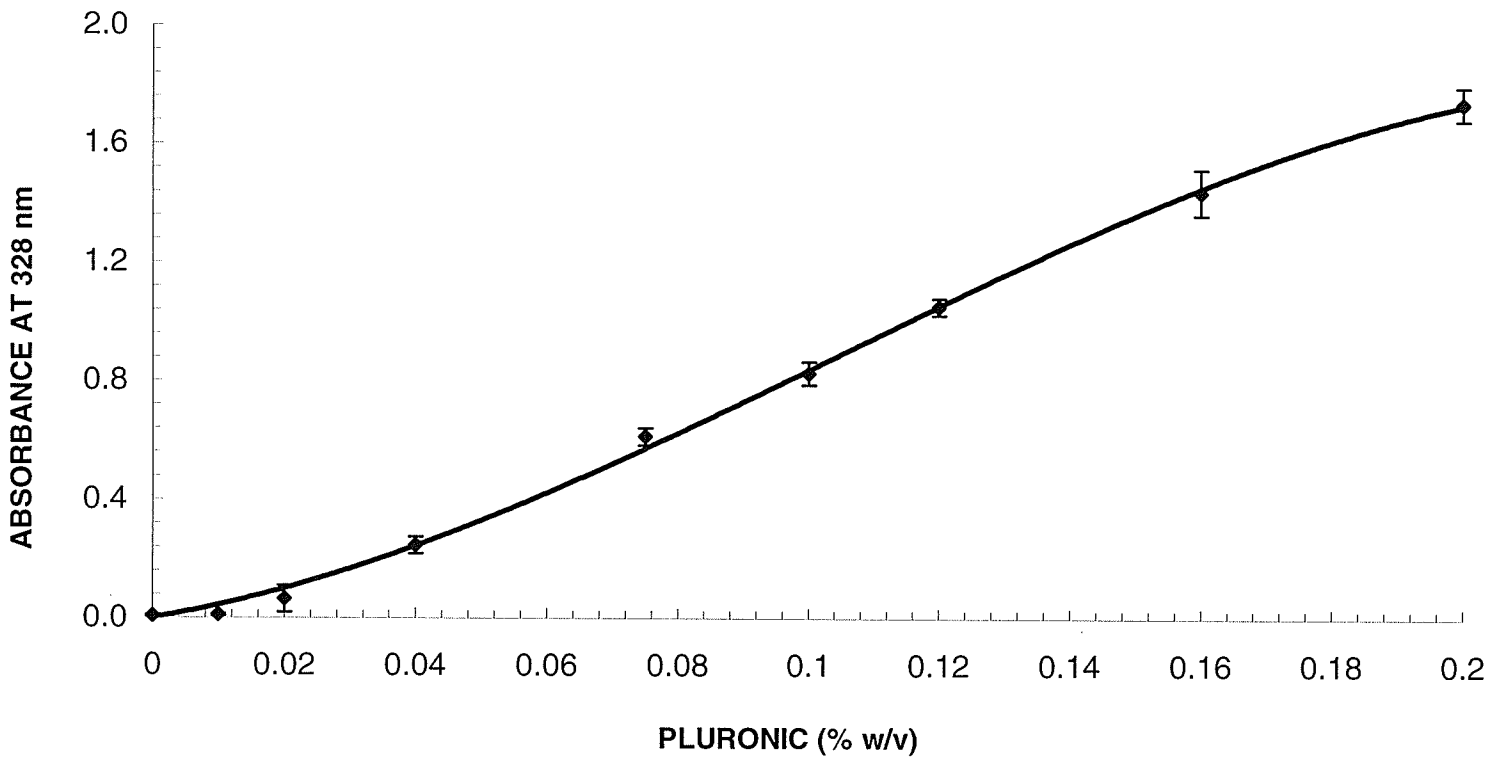


Figure 6.4 Standard curve for Pluronic F-68 in serum-containing culture medium (n=3, ±SD).

The protocol described in Fig. 2 (B) was followed for Pluronic F68 standards within the concentration range of 0.01-0.2% in serum-containing medium.

mixing and gas sparging. The modified assay described in this thesis may be used to quantify the presence of Pluronics during various upstream and downstream operations associated with mammalian cell culture technology. The assay may also be used for assessing intracellular accumulation and adsorption of Pluronic F-68 to gas-liquid and liquid-solid interfaces.

Conclusions:

- Increasing sample volume to 200 µl and using absorbance measurement of 328 nm increased the sensitivity of the assay around ten fold.
- The reproducibility of the assay was improved by washing the complex with ethyl acetate.
- The addition of a critical volume of ethanol was found to be necessary to reduce the interference caused by unknown components of the culture medium.
- The assay was linear between concentrations of 0.01% (w/v) and 0.2 % (w/v) in serum free medium.
- The presence of serum in the medium decreased the sensitivity of the assay, which nevertheless was linear from 0.04% to 0.16% (w/v) Pluronic F68.

Chapter 7 : Effect of Pluronic F68 on cell membrane characteristics

Previous results (chapter 4) showed that Pluronic F68 decreases the hydrophobicity index of mammalian cells. This decrease in the hydrophobicity index was interpreted (at least partially) as a decrease in the cell surface hydrophobicity of cells rather than a solely solvent solute interaction. In chapter 5 results showed that Pluronic F68 increased the robustness of cells (decreased sensitivity to shear forces), an effect that was not due to an increase in viscosity or other change in the physical properties of the culture fluid per se. Both effects were dose dependent in at least one cell line. All these effects suggest an interaction with the cell membrane. In this chapter we extend the work to test the ability of Pluronic F68 to affect some of the other cell membrane characteristics.

Effect of Pluronic F68 on linoleic acid uptake

Some nutrients (ex. linoleic acid) are taken up into the cell through a passive diffusion process. Molecules that interact with the cell membrane (like Pluronic F68) can affect the uptake of these nutrients. This effect might report on the nature and degree of Pluronic F68 interaction with the cell membrane. Pluronic F68 has been shown to decrease the hydrophobicity index of mammalian cells (chapter 4). Therefore an experiment was performed to test the effect of Pluronic F68 on the uptake of linoleic acid by CC9C10 cells using a ^{14}C-radiolabelled linoleic acid. Linoleic acid was chosen because of its hydrophobic nature as well as its passive diffusion uptake process.

Radiolabelled (hot) ^{14}C linoleic acid equivalent to 27 μCi dissolved in toluene was air dried and mixed with 3 mL of MilliQ water containing BSA, cyclodextrin (0.1 mg/mL), or nothing. Non-radiolabelled (cold) linoleic acid (3 μL) was added from a stock solution in ethyl alcohol (1 M). The solution was mixed for 1 hour at room temperature. The three different preparations of linoleic acid were then added to the medium at a ratio of 1 mL to 100 mL of medium giving a final concentration of 10 μM of cold linoleic acid and 1.8 μM of "hot" linoleic acid (total of 11.8 μM). CC9C10 hybridoma cells at density of 1.5 x 10^6 cells/mL were suspended in growth medium either with or without Pluronic F68 (0.1% concentration). In all cases the cells were incubated at 37° C and samples of 0.5 mL of homogenously suspended cells were taken every 5 minutes and put on a 0.2 μm filters under vacuum. The cells were trapped on the filter and the radioactivity of the filter was measured using liquid scintillation counting.

The cells trapped on the filters were washed with BSA (0.1%) in PBS before measuring the radioactivity. This step was important to remove the excess linoleic acid on the cell surface. Washing with PBS only was not effective in removing all the fatty acid adsorbed to the cell surface. This resulted in a high background reading equivalent to 1.5 nmole of radioactive linoleic acid when the free, un-complexed, linoleic acid was used in the medium. Therefore in all uptake experiments, excess unbound labelled linoleic acid was washed with 0.1% BSA in PBS (Appendix, Figure A.6).

Figure 7.1 shows the effect of Pluronic F68 on the uptake of linoleic acid complexed with BSA. There was no significant difference in the presence of Pluronic F68 from the

control. In both cases the total uptake was around 2.5 nmole per 10^6 cells and the rate of uptake was linear in the first 16 minutes with a rate of 80 pmole/10^6 cells/min (R^2 =0.99). Figure 7.2 shows the effect of Pluronic F68 on the uptake of linoleic acid complexed with cyclodextrin. Pluronic F68 significantly decreased the uptake rate of linoleic acid during the 60 min of the experiment. The uptake rate decreased from 111 pmole/10^6 cells/min to 85 pmole/10^6 cells/min while R^2 was 0.999 in the linear part of the uptake curve. Figure 7.3 shows the effect of Pluronic F68 on the uptake of free (un-complexed) linoleic acid. Pluronic F68 significantly decreased the absorption of linoleic acid although there was no significant difference between the total up-take of linoleic acid in either situation. The initial uptake rate decreased from 126 pmole/10^6 cells/min to 87 pmole/10^6 cells/min in the presence of Pluronic F68. Therefore we conclude that Pluronic F68 decreased the uptake rate of linoleic acid complexed with cyclodextrin or administered in a free form, while it had no significant effect on the uptake of linoleic acid complexed with BSA.

To compare between the different methods of delivering linoleic acid the uptake curves were re-plotted for the three conditions. Figure 7.4 shows such a comparison. The total linoleic acid concentration was 11.8 μM in each culture. Free linoleic acid and β-cyclodextrin complexed linoleic acid had significantly higher uptake rates than linoleic acid complexed with BSA, with initial uptake rates of 126, 111 and 80 pmole/10^6 cells/min respectively. This shows a significantly higher absorption rate of free linoleic acid and linoleic acid complexed with cyclodextrin compared with linoleic acid complexed with BSA.

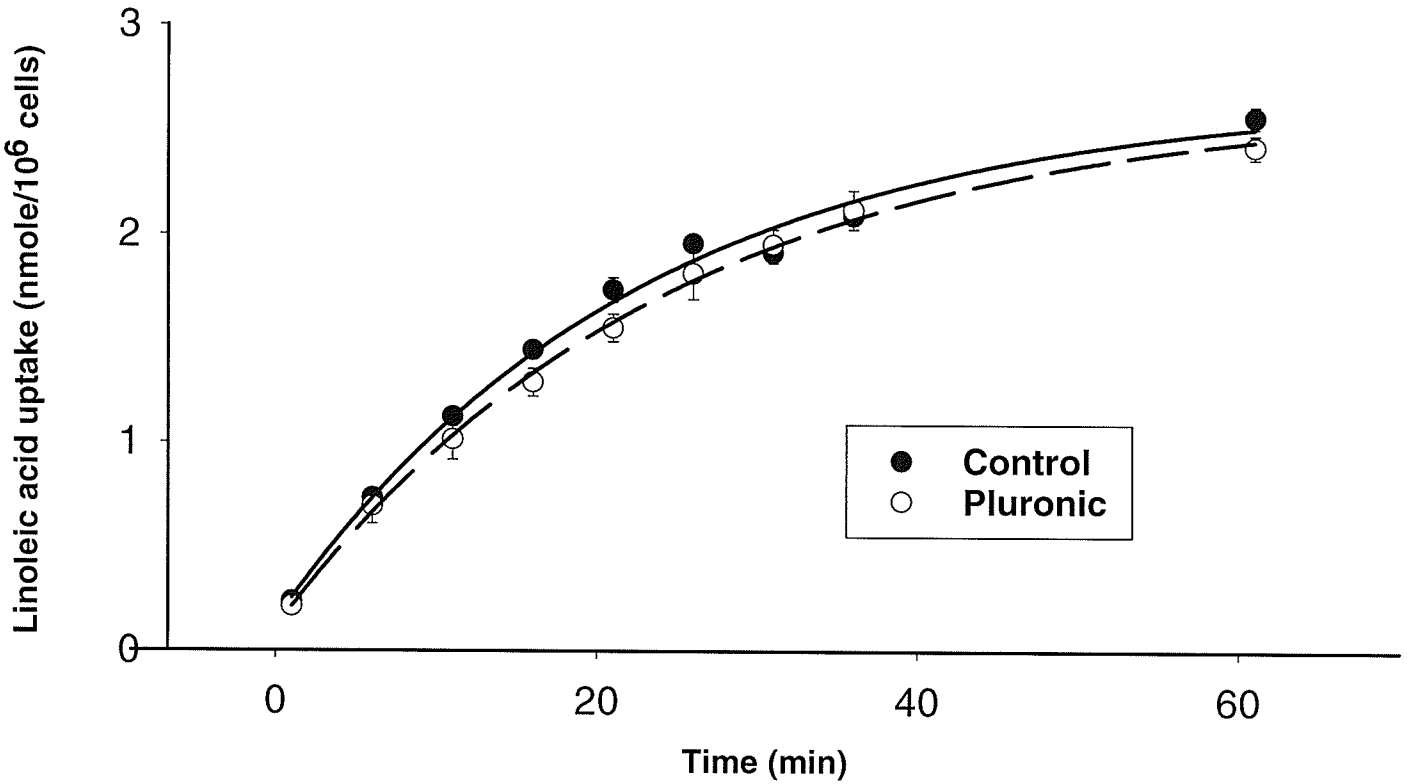


Figure 7.1 The effect of Pluronic F68 on the uptake of linoleic acid complexed with BSA at 37° C.

CC9C10 cells with a density of 1.5 x 10^6 cells/mL were incubated with linoleic acid (10 µM) mixed with ^{14}C-radio-labelled linoleic acid (1.8 µM) complexed with BSA (0.1 mg/mL) at 37 °C and in a shaker to mix the cells. Samples of well mixed cells were taken at 5 minutes intervals, trapped on 0.2 µm disc filters and washed with 0.1% BSA in PBS. Error bars represent Standard Error of the Mean (± S.E.M; n=4).

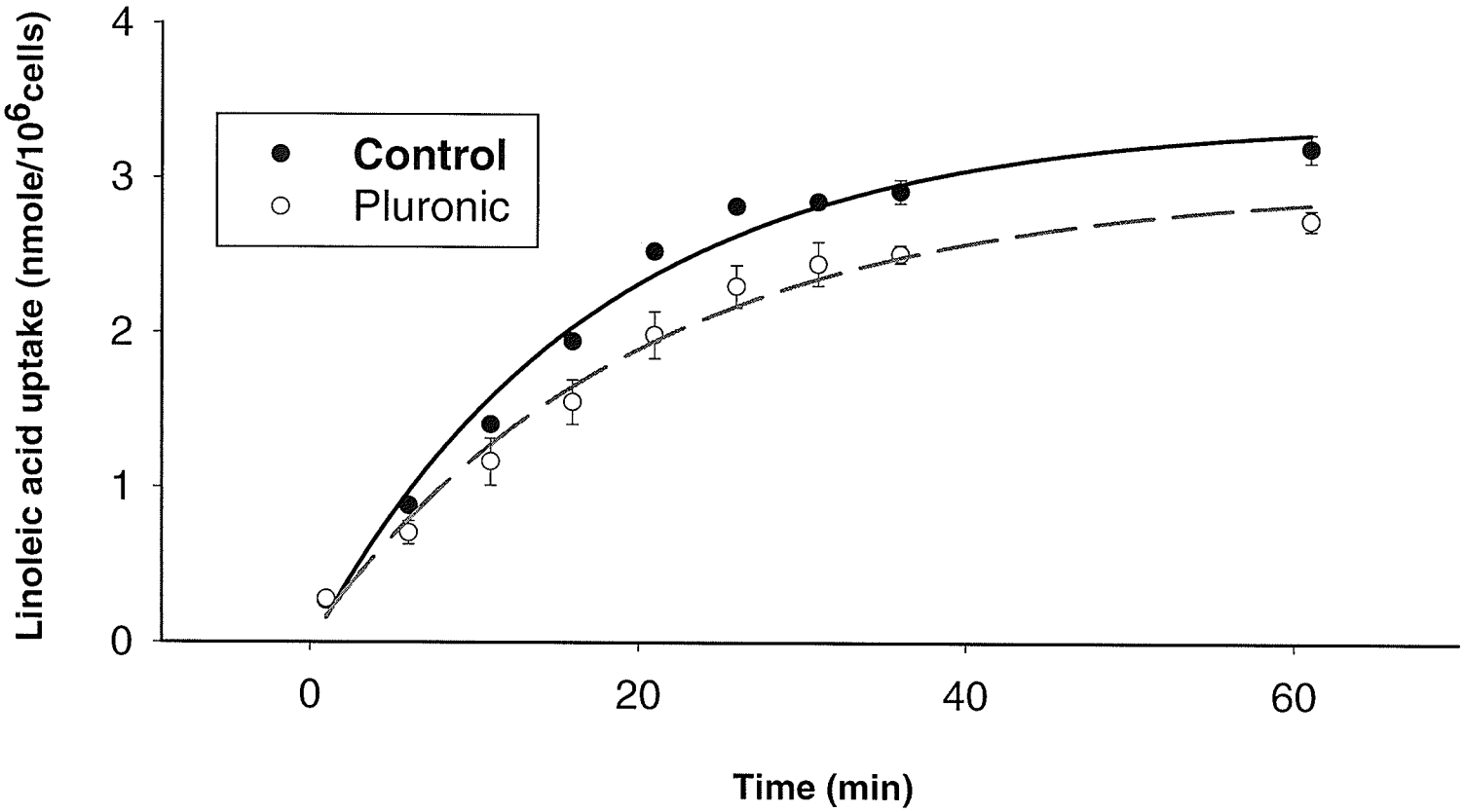


Figure 7.2 The effect of Pluronic F68 on the uptake of linoleic acid complexed with cyclodextrin at 37 °C.

The conditions are the same as above but CC9C10 hybridoma cells were incubated with linoleic acid complexed with cyclodextrin (0.1mg/mL) at 37 °C (n=4).

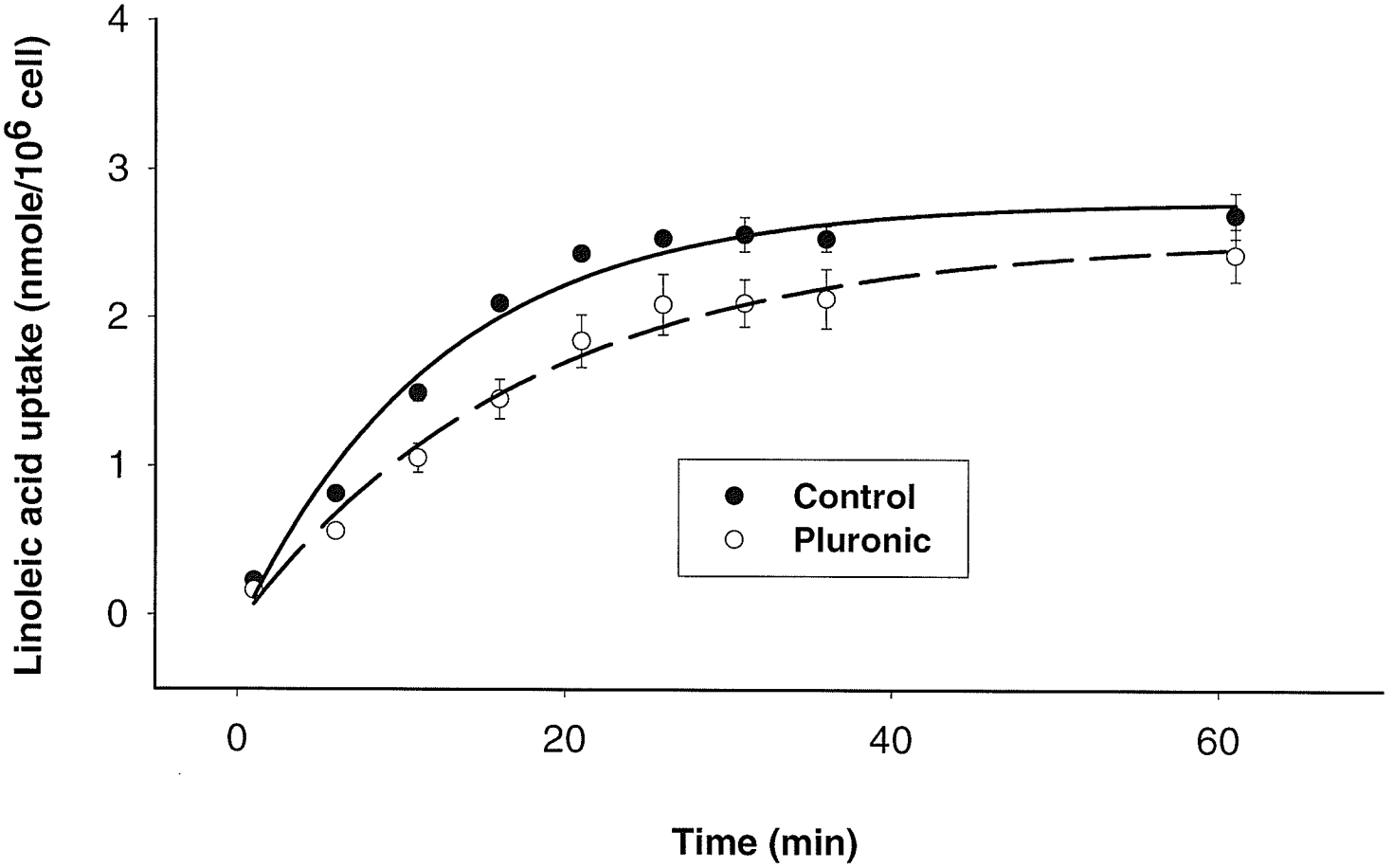


Figure 7.3 The effect of Pluronic F68 on the uptake of linoleic acid introduced as free in the medium at 37 °C.

The conditions are the same as above but cells were incubated with un-complexed linoleic acid that was dissolved by the aid of high pH (n=4).

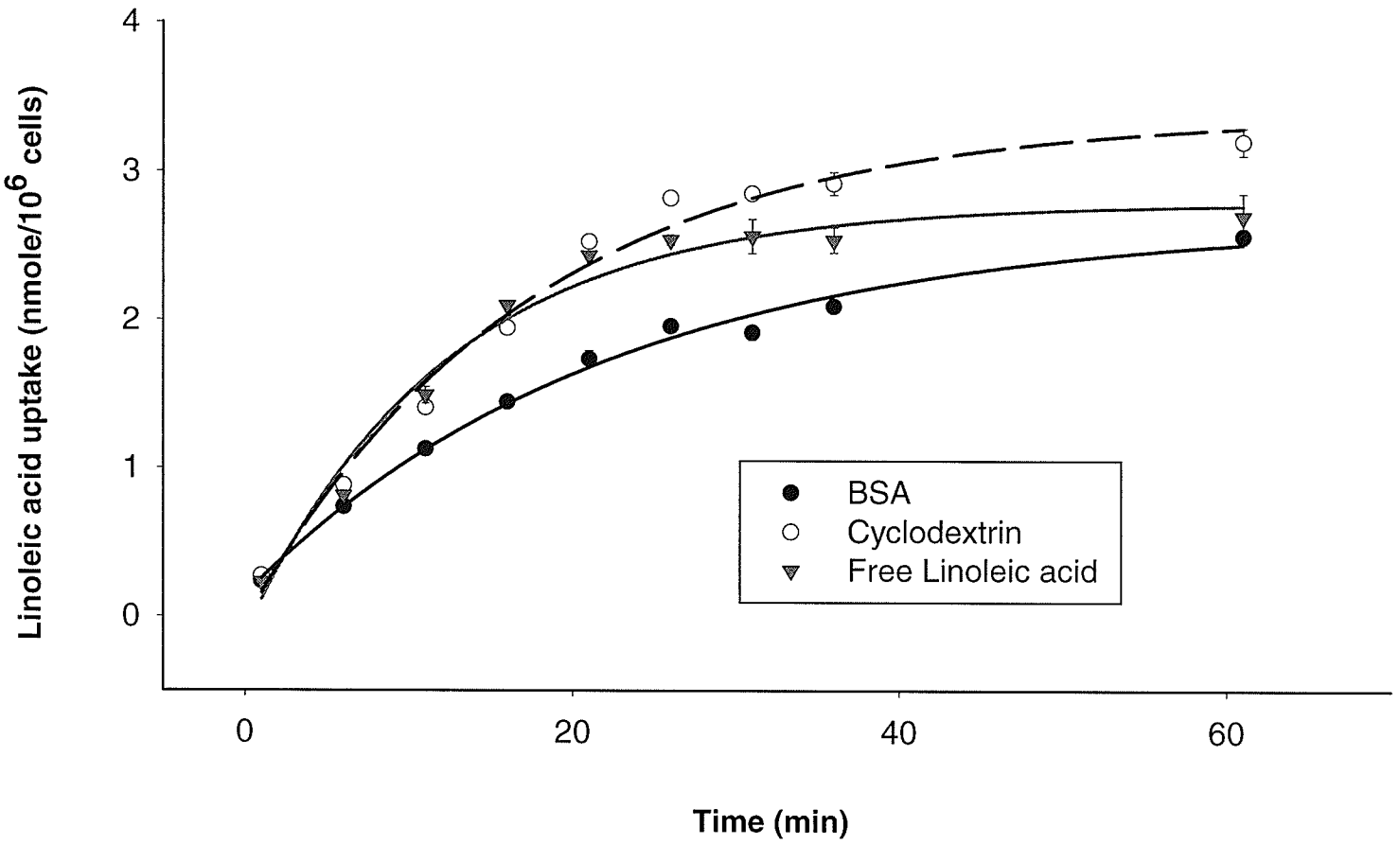


Figure 7.4 Comparison between the different ways of delivering linoleic acid to CC9C10 hybridoma cells.

The control curves of Figure 7.1, 7.2 and 7.3 were re-plotted together. Error bars represent S.E.M. (n=4).

Effect of Pluronic F68 on the Plasma Membrane Fluidity:

Plasma Membrane Fluidity (PMF) and Electron Spin Resonance (ESR):

Membrane lipids are in a "fluid" state. Plasma membrane fluidity (or micro viscosity) is the physical state of the membrane lipids due to motion of acyl chains around the fatty acids' axes and the rotational isomerisation around the carbon-carbon bonds. This fluidity is dependent on the membrane composition of fatty acids, cholesterol and other lipid molecules and the temperature. Many external compounds have been reported to change the membrane fluidity for example, alcohol, fatty acids, toxins...etc.

Information on the behaviour of the lipid molecules in cell membrane can be gained from the ESR signals of lipid spin labels. These labels are reporter molecules, which contain the nitroxide (Doxyl) group (Figure 7.5) giving characteristic signals (presented as spectra) that depends on both the molecular tumbling rate and the orientation of the label in the presence of an external magnetic field provided by the ESR instrument (Calder et al. 1994).

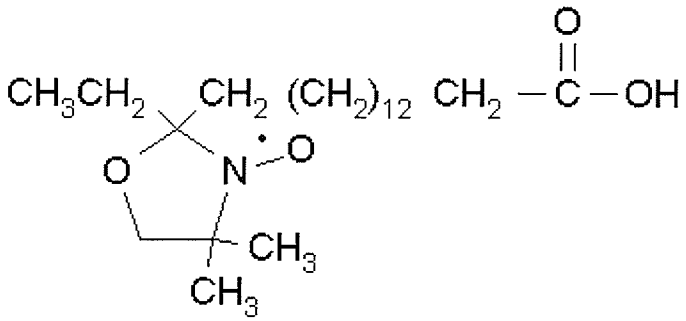


Figure 7.5 Chemical structure of 16-doxyl stearic acid spin label. 5-Doxyl spin label is the same except for the doxyl group which is present on the carbon 5 of the stearic acid.

ESR spectra of spin labels are therefore sensitive to molecular order, viscosity and polarity of the labels' surrounding environment. The orientation of these probes (labels) in the membrane is such that the long axis of the probe is parallel to the phospholipid fatty acids. By varying the position of the doxyl substitute on the fatty acid, different locations (depths) within the membrane can be examined. The 5-doxyl label probes the membrane bilayer near the surface whereas 16-doxyl monitors the core of the bilayer (Zaleska et al. 1989).

The order parameter is a measure of the amplitude of motion of acyl chains around the molecular axis from the average orientation of the fatty acid chain in the lipid bilayer. The maximum is 1 for perfect order (rigid axial motion) while complete disorder (anisotropic) results in a value of 0. The order parameter (S) can be calculated from ESR spectra according to equations (1) and (2) (Foucher et al. 1996).

$$S = 0.564 \times (A_{\parallel} - A_{\perp}) / a' \qquad (1)$$

$$a' = 1/3 \, (A_{\parallel} + 2 \, A_{\perp}) \qquad (2)$$

where $A_{\parallel}$ and $A_{\perp}$ are hyperfine splittings that can be obtained from the ESR spectra (figure 7.6). Decrease of the order parameter S or the parameter $A_{\parallel}$, or an increase of the parameter $A_{\perp}$ indicates a higher disorder or dynamics of the hydrophobic part of the membrane. The order parameter (S) calculation via equation 1 is appropriate at the 5 position in the upper portion of the fatty acid acyl chain, where molecular motion is sufficiently anisotropic to result in spectra from which outer and inner hyperfine extrema are discernible (Wassall, S. et al. 1992).

The rotational correlation times (T_c) are applicable for 16-doxyl probes, which report about the deeper part of the cell membrane where the acyl chain motion can be considered anisotropic. T_c is an empirical motion parameter that is valid only for rapidly tumbling units i.e. $< 10^{-9}$ s and it can be calculated according to the equation:

$$T_c = 6.5 \times 10^{-10} W_0 \left[(h_0/h_{-1})^{1/2} - 1\right] \qquad (3)$$

where W_0 is the peak to peak width of the central line, and the h_0/h_{-1} is the ratio of the heights of the central and high field lines, respectively (Curatola et al. 1985; Wassall, S. et al. 1992).

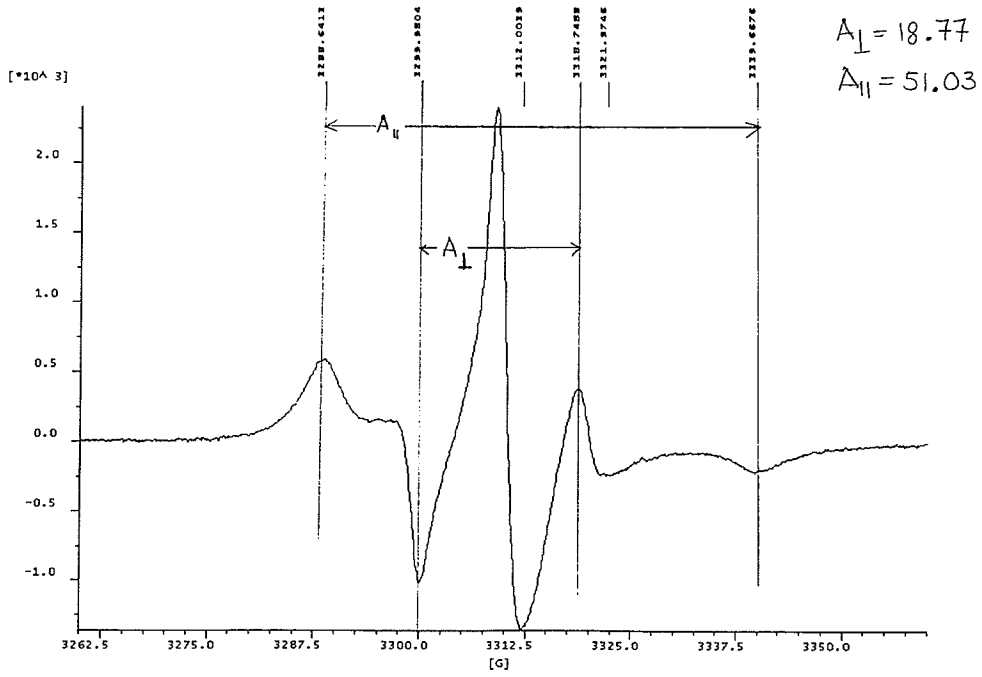


Figure 7.6 The ESR spectrum for 5-Doxyl stearic acid at 37 °C incorporated in CC9C10 hybridoma cells. $A_{\|}$ and $A_{\perp}$ are hyperfine splittings and they are measured in Gauss (G) and represent spectral parameters.

The ability of Pluronic F68 to be incorporated in the cell membrane or change the lipid/protein distribution would probably affect the plasma membrane fluidity (PMF) of cells. This has been shown previously on insect cells using 0.5% Pluronic F68 (Ramirez and Mutharasan 1990).

To investigate the effect of Pluronic F68 on the PMF of CC9C10 cells, the 5-Doxyl stearic acid spin label was used. As mentioned above, this label gives a characteristic signal and reports on the molecular order and viscosity of the upper portion (close to outside) of membrane. The signal was interpreted in terms of order parameter (S), which is defined as a measure of the amplitude of motion of the molecules axis about the average orientation of the fatty acid chain in the lipid bi-layer.

CC9C10 cells were cultured in the absence of Pluronic F68 and were re-suspended in PBS and incubated with 5-doxyl-stearic acid spin label. Cells were centrifuged and re-suspended in PBS containing Pluronic F68 with concentrations ranging from 0 to 0.5% Pluronic F68 in a capillary tube. The order parameter was measured by ESR and spectra were generated. The order parameter (S) was calculated from the equations (1) and (2). $A_{\|}$ and $A_{\perp}$ are hyper fine splittings (Figure 7.6) for the nitroxide label measured from the generated spectra (Curatola et al. 1985; Foucher et al. 1996).

Results are tabulated in Table 7.1 showing the effect of Pluronic F68 on the order parameter of CC9C10 hybridoma. All three measurements (n=3) were from the same batch of cells. Results show no significant difference in the order parameter of the

hybridoma cell membrane at different concentrations of Pluronic F68 up to 0.5%. The standard deviation of the three different measurements at each Pluronic F68 concentration was higher than the difference between the order parameters at the different Pluronic F68 concentrations.

Therefore we conclude that there is no significant effect of Pluronic F68 present in the medium on the PMF of cell membranes of CC9C10 cells.

Table 7.1 The effect of Pluronic F68 on the Order Parameter (S)

Pluronic F68	S1	S2	S3	S (Av)*	± SD*
0 %	0.621	0.606	0.624	0.617	0.0099
0.1 %	0.617	0.629	0.625	0.624	0.0060
0.2 %	0.624	0.620	0.624	0.623	0.0025
0.5 %	0.628	0.613	0.615	0.619	0.0080

* Sav is the average of calculated order parameters and SD is the standard deviation.

Discussion:

The most recent mosaic model of the cell membrane is a lipid bilayer. This bilayer is formed from phospholipids with polar heads and a hydrophobic tail (Stryer 1988). The phospholipids are arranged so that the polar heads are towards the outer part of the bilayer while the hydrophobic tail is towards the inside. Because of this core hydrophobic part of the membrane, the diffusion of compounds across the membrane depends on their hydrophobicity. Relatively hydrophobic compounds can passively diffuse through the membrane. Linoleic acid is a hydrophobic molecule that is taken up by passive diffusion

(Abumrad et al. 1998; Hamilton et al. 2001). Beside the passive diffusion there is also a carrier protein for the transport linoleic acid across the cell membrane in some cell types (Abumrad et al. 1999, Murota et al. 2001 and Keinfeld 2000)

Pluronic F68 decreased significantly the rate of linoleic uptake except when BSA was absent in the medium. In the presence of Pluronic F68 the free linoleic acid or linoleic acid complexed with cyclodextrin was taken up at a lower rate compared with the control. This is possibly due to the effect of Pluronic F68 on decreasing cell surface hydrophobicity, which is important for the passive diffusion of linoleic acid across the membrane. However, Pluronic F68 did not decrease the absorption of linoleic acid in the same extent as it did for CSH (chapter 4 results). This probably can be explained by the presence of carrier proteins for the transport of linoleic acid across the cell membrane and therefore it can be suggested that in the presence of Pluronic F68 the carrier protein can compensate for the decreased passive diffusion due to the lowered cell surface hydrophobicity by Pluronic F68.

In the presence of BSA this effect of Pluronic F68 on the uptake of linoleic acid is absent probably due to the higher affinity to BSA. The linoleic acid might have a lower affinity to cyclodextrin, which leads to a faster adsorption of the fatty acid to membrane and therefore higher rate of uptake. Once adsorbed to the cell membrane the fatty acid will be taken up to the cell. In the absence of a complexing agent, linoleic acid probably complexes with the cell membrane proteins, which happens very quickly, followed by uptake into the cell. The rates of uptake were 126, 111, and 80 pmole/10^6 cells/min for

free linoleic acid, linoleic acid complexed with cyclodextrin, and linoleic acid complexed with BSA respectively.

Alternatively it might be reasonable to propose that in the presence of BSA the transfer of linoleic acid from BSA to the cells is slower due to the ability of BSA to decrease the cells' hydrophobicity index, which reflects a decrease in CSH (chapter 4). This interpretation might explain the lower uptake rate in the presence of BSA of both the control and in the presence of Pluronic F68.

The effect of Pluronic F68 on the plasma membrane fluidity has been reported by Ramirez and Mutharasan (1990). Results in this chapter did not show any significant change in the PMF of cell membranes. The difference in the results can be due to the different method employed since electron spin resonance was used here while fluorometry was used by Ramirez and Mutharasan (1990). Furthermore, the effect was shown by Ramirez and Mutharasan (1990) only at a very high concentration of 0.5% Pluronic F68 that is rarely used in cell culture, suggesting a marginal effect that might be hard to detect at lower concentrations.

Another difference, which might also be very important, is the difference of the treatment of cells in the two cases. Ramirez and Mutharasan cultured the cells in the presence of Pluronic F68 and then re-suspended the cells into a Pluronic F68 free solution during the measurement. In our case Pluronic F68 was present during PMF measurement but the

cells in both the control and in the presence of Pluronic F68 were cultured in the absence of Pluronic F68.

Conclusions:

1. Pluronic F68 had no significant effect on the uptake rate of linoleic bound or complexed to BSA.
2. Pluronic F68 reduced significantly the uptake rate of linoleic acid bound to cyclodextrin or in free form.
3. Uptake rate of Linoleic acid bound to cyclodextrin or in free form is significantly higher than the uptake rate of linoleic acid complexed with BSA.
4. No significant effect of Pluronic F68 was detected on the Plasma membrane fluidity of CC9C10 hybridoma cells.

Chapter 8 The Molecular Interaction of Pluronic F68 with Cell Membranes: Pluronic Adsorption to cell membrane.

Pluronic F68 protects cells in culture by four main mechanisms (chapter 1), two of which involve interaction with the cell membrane while the other two are purely due to solvent-solute interaction (Michaels et al. 1995; Murhammer and Goochee 1990[b]). The Pluronic F68 interaction with cell membrane has been manifested by a decrease in the cell hydrophobicity index (Wu 1996; chapter 4), a decrease in the uptake of linoleic acid complexed with cyclodextrin or introduced free in solution (chapter 7), a decrease in the plasma membrane fluidity (Ramirez and Mutharasan et al. 1990), an alteration in the permeability of cell membrane (Al-Rubeai et al. 1993; chapter 7) as well as electric conductivity (King et al. 1991).

Although there are numerous results to suggest the interaction of Pluronic F68 molecules with the cell membrane, the nature and the property of this type of interaction is still not completely understood. The nature of this interaction was suggested before to be through an adsorption of Pluronic F68 molecules around the cell membrane (Marquis et al. 1989; Zhang et al. 1992), penetration and incorporation into the cell membrane (Zhang et 1992) and/or possibly changing the lipid/protein organization (King et al. 1991). This chapter is devoted to study the Pluronic F68 adsorption to cell membrane. In the light of the results in other chapters of the thesis previous suggestions on how Pluronic interact with cell membrane are discussed.

Pluronic F68 adsorption to cell membrane

To test for the possibility of Pluronic F68 molecules being adsorbed on the cell outer membrane we proposed that if Pluronic F68 molecules are adsorbed to the cell membrane, the concentration of Pluronic F68 in the cell culture medium will decrease. To measure the concentration of Pluronic F68 in cell culture medium an assay was required. None was available so we developed an assay (chapter 6) and we measured the concentration of Pluronic F68 in the cell culture medium. By measuring the concentration of Pluronic F68 in the medium the percentage of molecules adhering to the cell can be quantified. CHO cells with an inoculation density of 10^5 cells/mL were cultured over 4 days in a stationary T-flask. Daily samples were taken, centrifuged and the Pluronic F68 concentration in the supernatant was measured using the previously developed assay.

Figure 8.1 shows the concentration of Pluronic F68 during a 4-day culture of CHO cells. Although cell growth was typical with a maximum cell density of 7 x 10^5 cells/mL at day 4, the concentration of Pluronic F68 was constant showing no decrease of Pluronic F68 molecules from the medium. Therefore we concluded that either Pluronic F68 molecules were not bound to the cell membrane, or that only a small undetectable fraction of Pluronic F68 is adsorbed. The sensitivity of the assay was limited by a standard deviation of 0.017 (absorbance) at the 0.1% Pluronic concentration. Therefore changes in the Pluronic F68 concentration of more than 0.0017 % concentration (0.017 mg/mL) would be required to be detected by the assay.

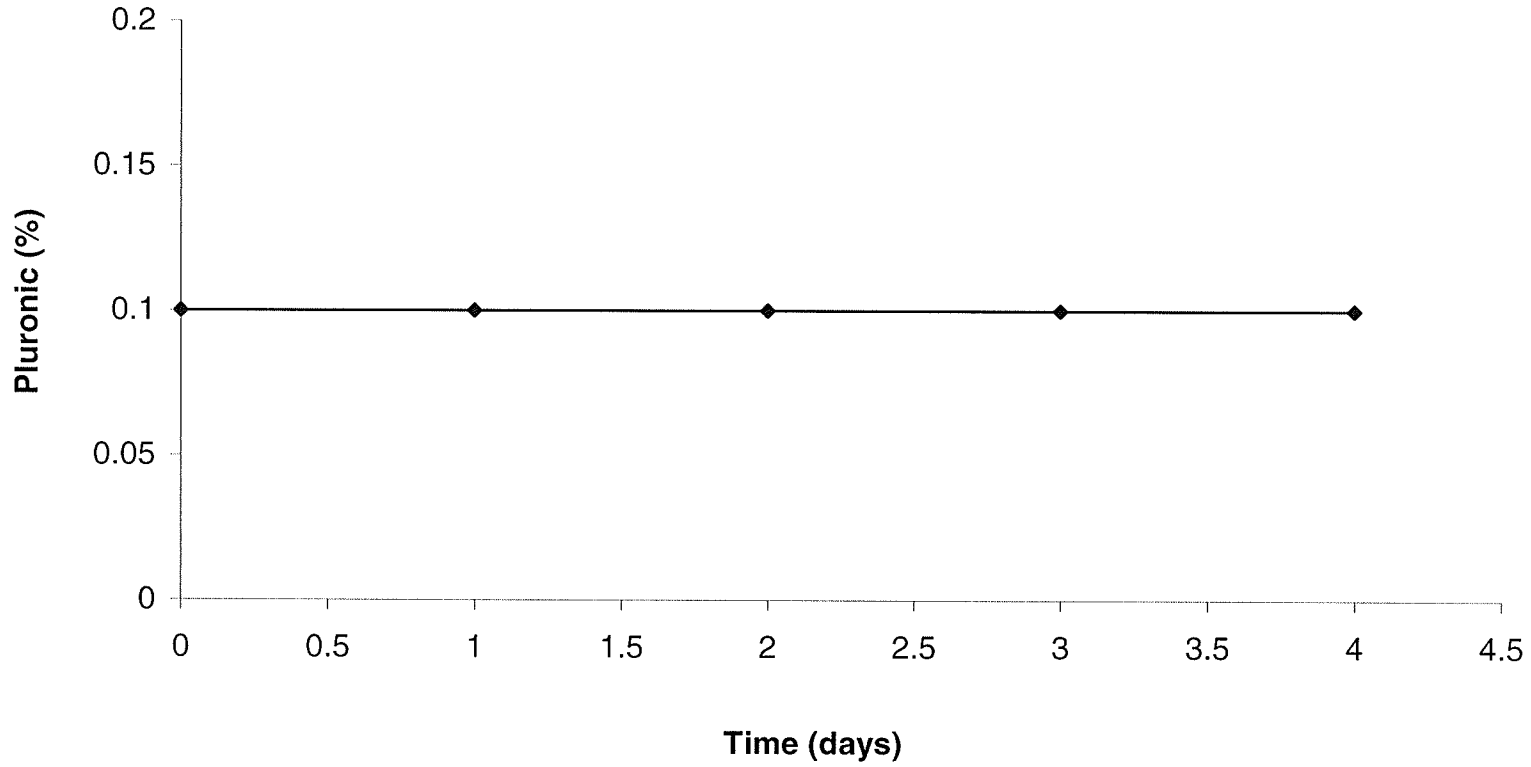


Figure 8.1 Pluronic F68 concentration in the culture medium of CHO cells.

CHO 81 cells were cultured for 4 days in a stationary T-flask from an inoculation density of 10^5 cells/mL to a maximum cell density of 7 x 10^5 cells/mL. Samples were taken daily and were analyzed for Pluronic F68 using a developed assay that depends on complexing Pluronic F68 with cobalt thiocyanate (n=1).

Calculating Pluronic F68 total surface area

There was no decrease in the dissolved portion of Pluronic F68 in the medium as measured by the assay. However, it was unknown if a small undetectable (by the assay) percentage of Pluronic F68 molecules interact with the cell membrane. To estimate the percentage of Pluronic F68 molecules that could possibly adsorb to the cell membrane – assuming 100% adsorption rate- two simplified approaches were attempted.

The first approach was to manually calculate the Pluronic F68 surface area that could possibly interact with the cells. However, to get an accurate figure of the Pluronic F68 molecular surface area calculations would be lengthy and they would be out of the scope of this thesis. For simplification and to get an approximate number it was assumed the following:

- The molecule is in a helix conformation when adsorbed to the cell based on information about Pluronic F68 in crystalline form and in aqueous solutions (Guo et al. 1999).
- Pluronic F68 molecules form a 7/2 helix (2 turns per 7 monomers) based on previous information from the literature about the polyethylene oxide helix in crystalline form (Figure 8.2; Takahashi et al. 1973).
- The molecules are packed on the cell surface side by side (100% adsorption rate).

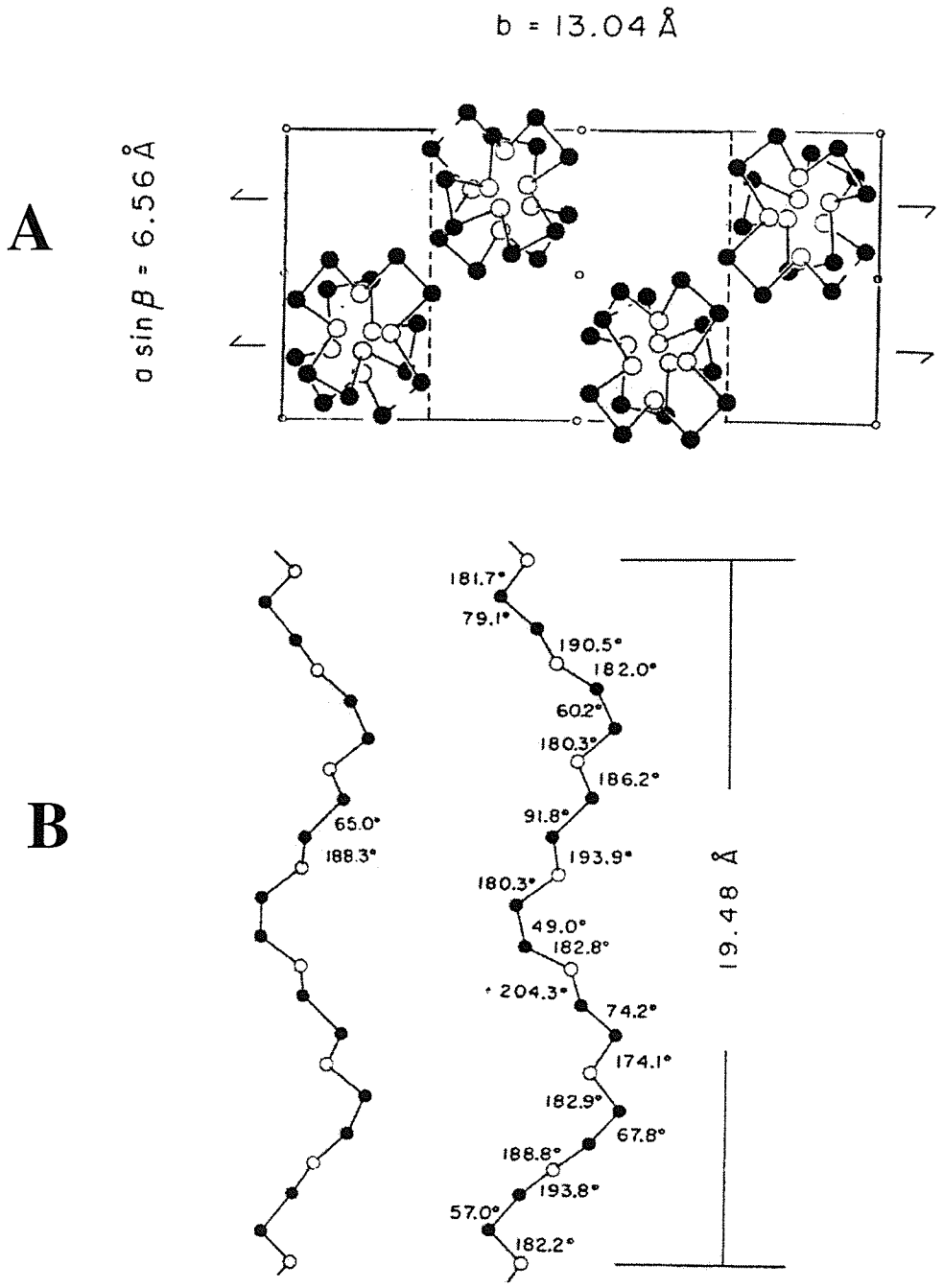


Figure 8.2 Helical structure of polyethylene oxide.

A) Crystal structure of polyethylene oxide. B) Molecular structure of polyethylene oxide with 7/2 helix conformation (Takahashi et al. 1973).

The second approach was to use software that can estimate the molecular surface area like Chem-3D (CambridgeSoft Corporation). The provided surface area is the Connolly surface area of the molecule, which is the contact surface, created when a spherical probe (representing the solvent) is rolled over the molecular model. This surface area does not represent the surface area of the molecule that can adsorb to a surface like the cell membrane but rather give an extended surface area that covers all the atoms and the bonds of the molecule (Figure 8.3a). In this situation an area like a footprint is required because the cell is much larger than the Pluronic F68 molecule and the cell membrane cannot adsorb to the hidden parts of the Pluronic F68 molecules due to steric hindrance. For simplification purposes, these areas obtained by the software were divided by 4 assuming the molecule is like a long helix that can fit into a rectangular prism with four major sides (top, bottom, back and front) and two relatively negligible ends (Figure 8.3b).

I) The Manual Calculation Approach:

According to Takahashi et al. (1973) in polyethylene oxide crystals each 2 turns per 7 monomers has a length of 19.48 Å and a width of 3.26 Å (assuming they are packed side by side which is not feasible in reality due to crystalline structure). Assuming a rectangular prism with a length of 19.48 Å and a width of 3.26 Å the surface area of one side of 7 monomers would be:

$$\mathbf{19.48 \times 3.26 = 63.5\ \text{Å}^2}$$

Pluronic F68 has generally a total of 180 units of ethylene oxide (and propylene oxide) per one molecule (for simplification the bottom surface area of the propylene oxide is

considered the same as the ethylene oxide which is not true in reality due to the extra methyl group) therefore the total of bottom surface area will be:

$$\mathbf{180 \div 7 \times 63.5 = 1632.9\ \text{Å}^2}$$

Since in 1 mL of medium with (1 mg/mL = 0.1% concentration) Pluronic F68 there are 1.19×10^{-7} moles (10^{-3} g of Pluronic/8400 (molecular weight)). In every mole there are 6.023×10^{23} molecules then there is total of 7.17×10^{16} molecules of Pluronic F68 in 1mL of medium. The total surface area of one side of Pluronic F68 molecules will be

$$\mathbf{1632.9 \times 7.17 \times 10^{16} = 11.7 \times 10^{19}\ A^2 = \underline{11.7} \times 10^5\ mm^2} \qquad [4]$$

II) The Software Calculated Surface Area:

The Chem 3D software (Cambridge Software) has calculated the Pluronic F68 Connolly molecular surface area to be 9300 Å^2, which is equal to

$$\mathbf{9300 \times 7.17 \times 10^{16} = 66.7 \times 10^{19}\ \text{Å}^2 = 66.7 \times 10^5\ mm^2}$$

for the sum of all Pluronic F68 molecules in 1mL at 0.1% concentration. By dividing the Connolly molecular surface area by 4 (assuming the molecule can fit into a rectangular prism with 4 major sides; Figure 8.3b) the surface area, for one side of the molecule, would be

$$\mathbf{66.7 / 4 = \underline{16.7} \times 10^5\ mm^2} \qquad [5]$$

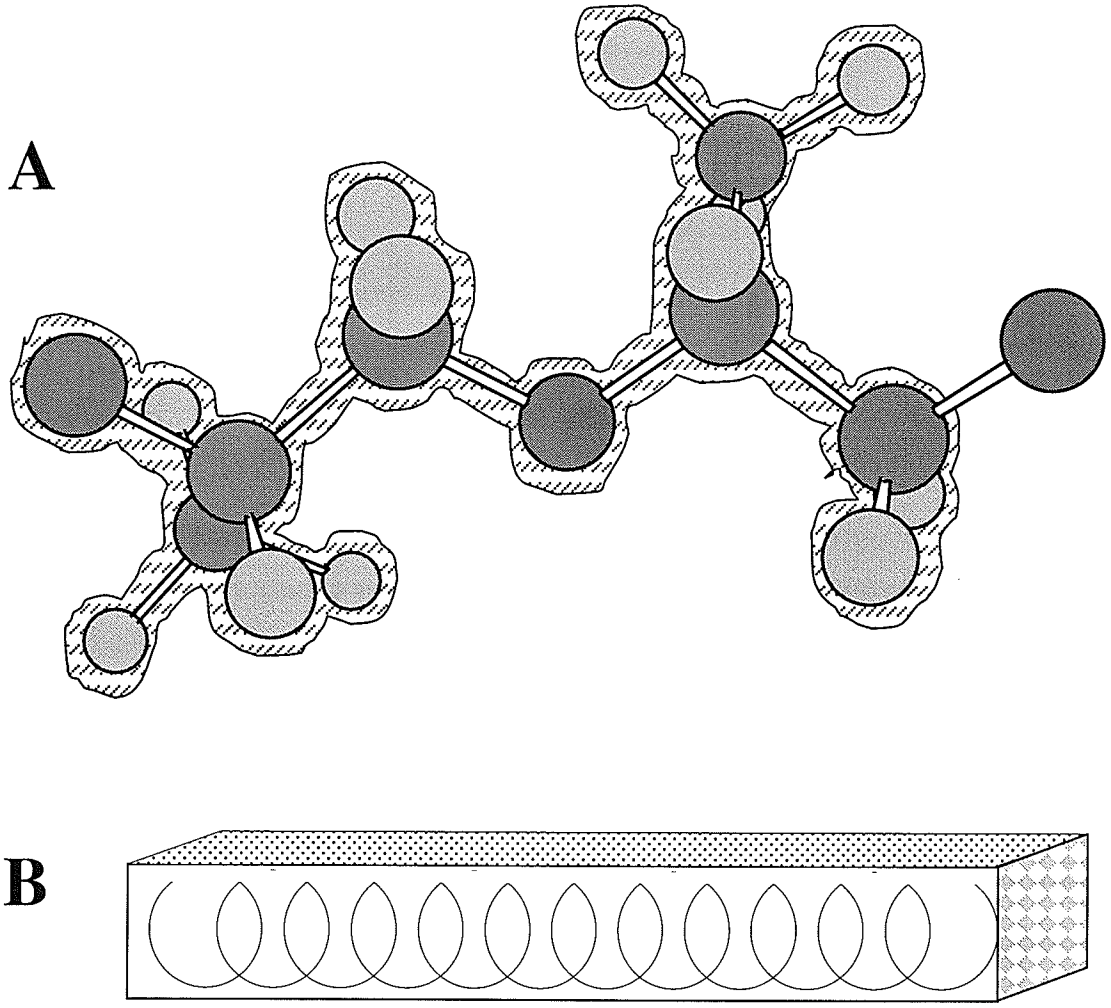


Figure 8.3 Calculation of Pluronic F68 surface area **(A)** Structure of two monomers of polyethylene oxide in a Pluronic F68 molecule as modeled by the Chem 3D software. Carbon atoms are shown in grey, oxygens in red, and hydrogens in blue. The dashed area around the molecules represent the Connolly surface area calculated by the Chem3D software. **(B)** For simplification an imaginary rectangular prism is drawn around the helical molecule (Pluronic F68) to show the 4 major sides (top, bottom, front or back) of the molecule with a two negligible ends (shaded with gray squares). Any of the four major sides can theoretically adsorb to a surface like the cell membrane.

III) The cells surface area calculations:

The average cell diameter of most animal cells is around 20 μm. The surface area will be equal to:

$$\pi r^2 = 3.14^* 100 = 314\ \mu m^2$$

In 1 mL of cell culture with a density of 10^6 cell/mL the total surface area of all the cells will be equal to:

$$314 \times 10^6 = 3.14 \times 10^2\ mm^2 \text{.....[6]}$$

Assuming that the cells will be covered completely with Pluronic F68 molecules we can divide the total available surface area from equation [6] over the total surface area of Pluronic molecules (equation [4] or equation [5]). The results show that cells in 1 mL of medium cannot take more than 2.68×10^{-4} or 1.68×10^{-4} (fraction, i.e. 2.68×10^{-2} or 1.68×10^{-2} %) of Pluronic F68 molecules which is equal to 2.68×10^{-4} mg or 1.68×10^{-4} mg because in every mL there is 1 mg at 0.1% concentration).

Therefore and regardless of the accuracy of the molecular surface area estimations of the Pluronic F68 molecules the large difference between the available surface area on the cell membrane and Pluronic F68 molecular surface area shows that cells cannot accommodate more than a very small portion of Pluronic F68 molecules unless there are multiple layers of Pluronic F68 or only a very small portion of each molecule is adsorbed to the cell.

We conclude that the quantity of Pluronic F68 that could be adsorbed by the available cell surface is below the detection limit of the assay. As mentioned before 0.017 mg/mL (or 17 μg/mL) change of concentration is the minimum to be detected

by the assay and this minimal change is far away from the maximum quantity of Pluronic F68 (2.68×10^{-4} mg/mL or 0.27 µg/mL) that could be adsorbed by the cells as a single monolayer.

Discussion:

Pluronic F68 has been shown to protect cells in bioreactors mainly by decreasing cell-to bubble attachment (chapter 4) and also possibly by strengthening the cell membrane (Zhang et al. 1992; chapter 5). Pluronic F68 also decreased the uptake of linoleic acid complexed with cyclodextrin or introduced free in the medium. However it is unknown how this non-ionic surfactant interacts with cells to produce its effect. Earlier studies have proposed that Pluronic F68 coats the cells with a viscous layer of polymer (adsorption) which buffers them from hydrodynamic forces (Marquis et al. 1989) while others suggested an alteration of the protein lipid interactions (King et al. 1991). Zhang et al. (1992) suggested that Pluronic F68 molecules are adsorbed to the membrane outer surface, a fast process, and incorporated, a slow process, in the inner part of the membrane.

In this chapter the results show that there was no measurable decrease in the concentration of Pluronic F68 in the growth medium. Calculations of the total available cellular surface area show that it is not enough to accommodate more than a very small fraction of the total dissolved Pluronic F-68 on the cell membrane. Other researchers have measured the percentage of Pluronic F68 that is adsorbed to the cell membrane of insect cells using a different assay (Wu et al. 1997). The adsorbed percentage of Pluronic

F68 was calculated by subtracting the amount of Pluronic F68 remaining in solution from the originally available amount after mixing cells with Pluronic F68 in PBS. They have shown extensive adsorption with low concentrations ranging from 25 to 200 μg/mL. They also used a mathematical model (Langmuir isotherm, $X = \frac{X \max KC}{1 + KC}$, where X= adsorbed amount, C is the concentration and K is a constant), which predicts a complete saturation at concentrations of 0.1% (1000 μg/mL) to 0.2% (2000 μg/mL). Concentrations used in this thesis are close to those that the Langmuir isotherm predicted for maximum saturation. However, even at close to saturation only very small fraction of Pluronic F68 is adsorbed as shown by in this thesis (undetected by the assay) and as predicted by Langmuir isotherm (< 5 μg Pluronic/ 10^6 of Tn-High 5 insect cells).

In a monolayer, less than 0.27 μg of Pluronic F68 per 10^6 cells were expected from the calculations of Pluronic molecular surface area. However, 4.5 μg/10^6 cells were practically found by Wu et al. (1997). This difference could be due to multiple layers of Pluronic F68 adsorbed and/or due to inaccuracy of the assay reported by (Wu et al. 1997) and/or the calculation of Pluronic surface area. The adsorbed amount of Pluronic F68 (4.5 μg/mL) was also below the minimum that can be detected by the assay developed in chapter 6 (16 μg/mL).

Another possibility is that only a portion of each molecule (ex. The hydrophobic one) is adsorbed to the cells. This has been shown with Pluronic F68 and polymeric colloids. The polypropylene oxide chain of the molecule anchored to the polystyrene spheres while the

polyethylene oxide chains extended into the aqueous environment (Li et al. 1994; Figure 8.4). If this is true with Pluronic F68 interaction with mammalian cells then the possible amount of Pluronic F68 adsorbing to the cell surface calculated previously (0.27 μg / 10^6 cells) can then be multiplied by 180 (number of total monomers in each molecule) and divided by 30 (number of polypropylene oxide monomers only). The result is 1.62 μg/ 10^6 cells which is closer to amount predicted by Wu et al. (1997).

Although Wu et al. (1997) showed the adsorption of Pluronic F68 to cell membranes this does not explain the ability of Pluronic to change some of the cell membrane characteristics. This is because the adsorption happens mainly at concentrations between 25 to 200 μg/mL of Pluronic F68. Increasing the Pluronic concentration from 25 μg/mL to 1000 μg/mL only gave an increase in adsorbed Pluronic F68 from 4 to 4.5 μg/mL/10^6 cells. On the other hand, changing the Pluronic F68 concentration from 25 to 200 μg/mL shows only a minor effect on the robustness of hybridoma cells (chapter 5). Rather higher concentrations were required (200 to 1000 μg/mL) to show maximum effects. Furthermore, a higher concentration of 200 to 2000 μg/mL was needed to decrease the cell surface hydrophobicity of CHO cells. Therefore the amount adsorbed of Pluronic F68 does not correlate with the effect of Pluronic F68 on mammalian cells. This suggests that it is less likely that adsorption of Pluronic F68 per se is the mechanism behind Pluronic F68's effect on membrane surface hydrophobicity (chapter 4), membrane robustness (chapter 5), and the uptake of nutrients (chapter 7).

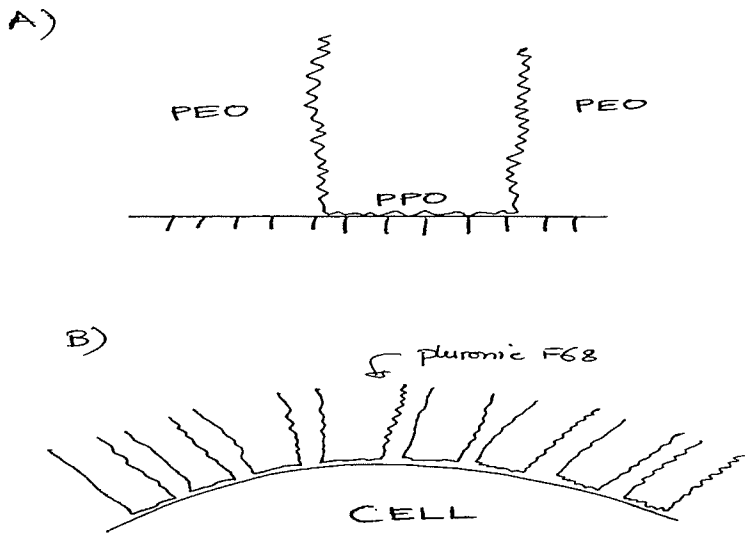


Figure 8.4 A) Adsorption of Pluronic F68 to polystyrene spheres. Polypropylene oxide (PPO) units adsorb to the cell while polyethylene oxide (PEO) flanks extend out in the medium. B) Pluronic F68 adsorption "side by side" to the cell membrane (Li et al. 1994).

A change in lipid/protein interaction is possible. However, a change in plasma membrane fluidity was not detected by ESR (chapter 7). Furthermore a change in lipid/protein interactions would probably require a longer time which was not the case in the effect on the uptake of linoleic acid (chapter 7), the cell surface hydrophobicity (chapter 4) and shear sensitivity of cells (chapter 5).

Another explanation that might be more promising is the change of the electrostatic charges around the cell membrane environment. This might explain the alteration of

permeability of cell membrane to fluorescein dye as shown by Al-Rubeai et al. (1993), the change in the electric conductivity (King et al. 1991) as well as the changes in the linoleic acid uptake and the hydrophobicity index of cell membranes (chapter 5 and 7). This hypothesis however has to be tested by further investigations.

Conclusions:

1. The Pluronic F68 concentration in the medium does not change over time during cell culture as measured by the developed assay in chapter 6.
2. According to calculations total available surface for Pluronic adsorption cannot accommodate more than 0.27 μg of Pluronic F68 in 1 mL of 10^6 cells suspension unless there is overlapping of these molecules.
3. According to calculations if Pluronic F68 adsorbs to the cell similarly to its adsorption to polystyrene spheres then the total surface area of cell membrane can accommodate 1.62 μg of Pluronic F68 in 1 mL of 10^6 cells suspension.
4. With the available data the adsorption of Pluronic F68 to the cell membrane cannot explain all changes made by Pluronic F68 on the cell membrane.

Chapter 9 : The protective effect of Linoleic acid and BSA on mammalian cells.

Serum has proved to be protective to cells in sparged large-scale animal cell culture (Ramirez and Mutharasan 1990; Van der Pol et al. 1992; Martens et al. 1992; Croughan et al. 1989; Handa-Corrigan et al. 1989; Michaels et al. 1991; Kunas et al. 1990; Jordan et al. 1994; McQueen et al. 1989). However serum use carries many unwanted problems such as batch variance, it is chemically undefined, and is quite expensive (Butler 1990). To avoid these disadvantages many attempts have been made to remove serum and substitute it with other additives. However, removal of serum from cell culture medium is complicated as it performs many biological functions in cell culture.

Linoleic acid is one component of serum that some mammalian cells are not able to synthesize, thus it is designated an essential fatty acid. Linoleic acid in serum free media has been shown to increase the robustness of cells under high shear forces (Butler et al. 1999) and lipids of serum in general have been shown to affect CHO cell growth and recombinant protein production (Castro et al. 1995).

Bovine serum albumin (BSA) is another component that has been used frequently in serum free media (Barnes et al. 1980; Castro et al. 1996). BSA in serum free media has been shown to protect cells from shear stress (Butler et al. 1999) and protect the glycosylation and production of recombinant proteins from deterioration upon serum

removal (Castro et al. 1996). BSA is a carrier molecule that complexes non-water soluble nutrients like fatty acids and cholesterol in culture media.

This study explores the effect of μM concentrations of cis-unsaturated linoleic acid supplements on the cell growth, cells robustness as well as cell membrane characteristics like hydrophobicity and membrane fluidity of CC9C10 hybridoma and CHO 81 cells. The effect of BSA on cell robustness is also investigated. The role of BSA on cell hydrophobicity has been discussed previously in this thesis (chapter 4).

1. Linoleic acid administration to the medium:

Linoleic acid is insoluble in water. There are several methods available to introduce it to the growth medium. The most widely used is dissolving in ethyl alcohol and introducing it into the medium as a concentrate in a water/alcohol mixture. Other methods depend on complexing with albumin (Butler et al. 1995) or cyclodextrin (Yamane et al. 1982, Gorfien et al. 2000), or solubilization with sodium hydroxide by converting into sodium linoleate. In order to choose the appropriate method we set up an experiment to test different methods of introducing linoleic acid into the growth medium.

Linoleic acid was stored as a non-sterile 1 Molar concentrate dissolved in absolute alcohol at –20 °C. The linoleic acid stock (3 μL or 20 μL) was then added to 10 mL distilled water, BSA (10 mg/mL), or Pluronic F68 (100 mg/mL) solutions and then filter sterilized. Linoleic acid was complexed with BSA using the previously published method (Butler et al. 1997). Briefly, the linoleic acid and 10 mL of BSA (10 mg/mL) solution

were shaken at 300 rpm for 1 hour and then filter sterilized. BSA used in this thesis was fatty acid free. Complexation of linoleic acid with Pluronic F68 was performed in a similar way. Linoleic acid in alcohol was made by dissolving linoleic acid in 60% ethyl alcohol in water followed by filter sterilization. The sterile concentrates were then diluted in the growth medium to give the desired final concentration of either 3 μM or 20 μM. Hybridoma CC9C10 cells were inoculated at 10^5 cells/mL and cultured in Biogro H medium in 9 x 25 cm^2 T-flasks for three days with a total culture volume of 8 mL. All cultures were established in duplicates (n=2). Each of the 9 T-flask cultures had one of the following conditions:

- No linoleic acid added to the medium (control).
- Pluronic F68 (1 mg/mL) added.
- Linoleic acid added without complexation (LA only).
- Linoleic acid dissolved in alcohol filtered and then introduced in the medium (LA + Alcohol).
- Linoleic acid complexed with Pluronic F68 in a similar way to BSA.
- Linoleic acid dissolved with a few drops of 4 Molar Sodium Hydroxide solution (LA + NaOH) either in the presence of Pluronic F68
    - or in the absence of Pluronic F68.
- Linoleic acid complexed with BSA either in the presence of Pluronic F68
    - or in the absence of Pluronic F68.

Figure 9.1 shows the cell yield of CC9C10 cells in the presence of 3 μM linoleic acid introduced to the medium in different ways. Results show that all conditions gave similar cell yields except for a lower one by linoleic acid introduced as sodium linoleate. The control achieved a cell yield of (14.9 ± 0.9) x 10^5 cells/mL while cells with linoleic acid dissolved by sodium hydroxide achieved (11.5 ± 0.9) x 10^5 cells/mL.

In Figure 9.2 a high concentration of 20 μM linoleic acid was used. The cell yields from cultures supplemented with linoleic acid dissolved by alcohol, solubilized by sodium hydroxide or complexed with Pluronic F68 were significantly lower. Cell growth halted and became non-viable within a couple of days (Figure 9.2). No inhibitory effect was detected by linoleic acid complexed with BSA with cell yields of (13.8 ± 0.69) x 10^5 cells/mL with growth close to the control of (14.8 ± 0.22) x 10^5 cells/mL. Therefore the inhibitory effect is dependant on the concentration and the way LA was introduced to the medium.

Linoleic acid added alone was most probably retained in the filter (during sterilization process) because it is insoluble in water. On the other hand, when complexed with Pluronic F68, high linoleic acid concentration was significantly inhibitory to cells. Because of the protective /neutralizing effect of BSA, linoleic acid was complexed with BSA as a carrier molecule for further experiments.

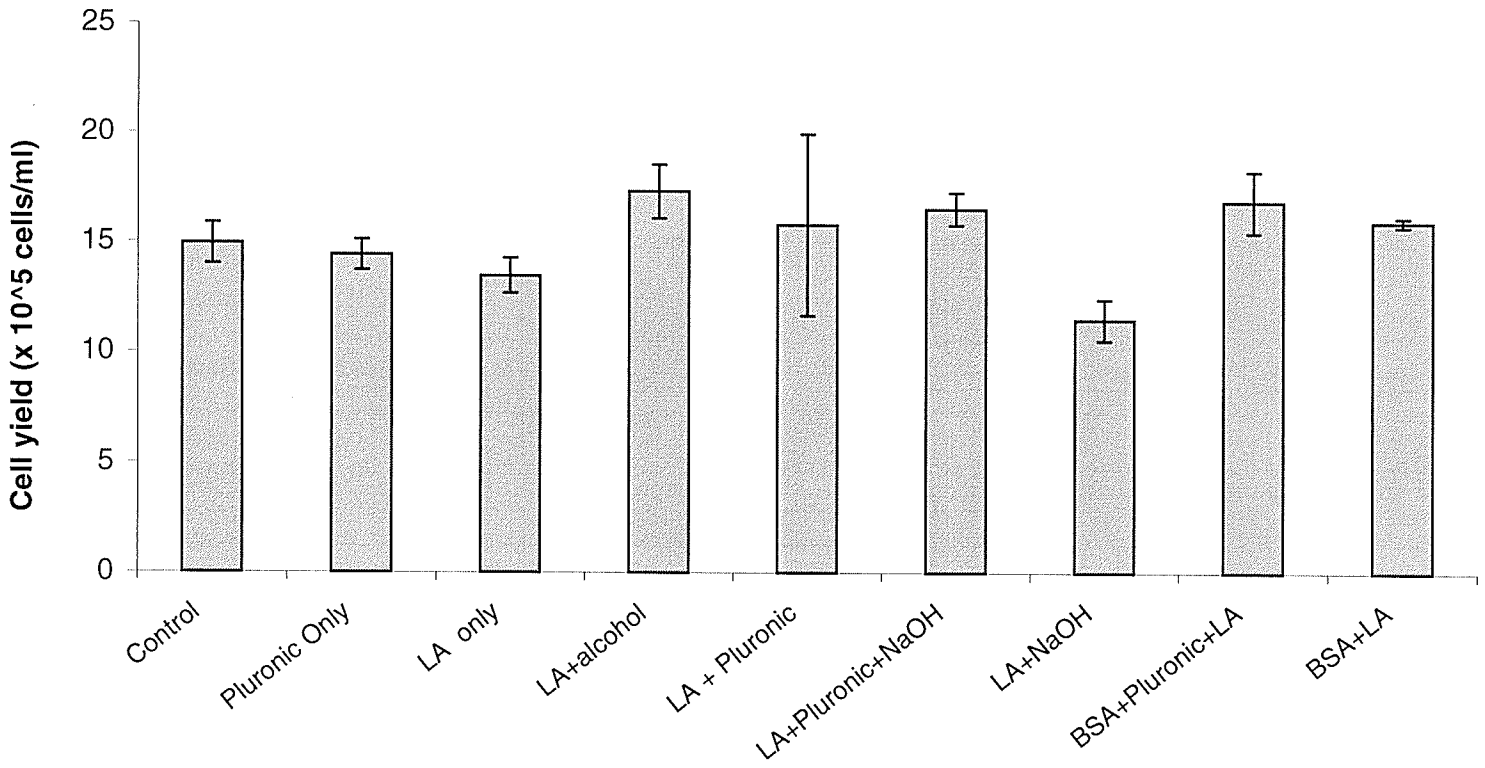


Figure 9.1 The effect of 3 μM linoleic Acid introduced differently on the growth of hybridoma CC9C10 cells.

Cells were cultured in 25 cm^2 stationary T-flasks for 3 days. Error bars represent standard deviation, (n=2). Inoculation density was 10^5 cells/mL. Linoleic acid stock was prepared by adding linoleic acid in alcohol to 10 mL of distilled water or 60% alcohol. In the case of distilled water, LA was complexed with Pluronic F68, BSA or dissolved with the aid of sodium hydroxide (NaOH) followed by sterile filtration.

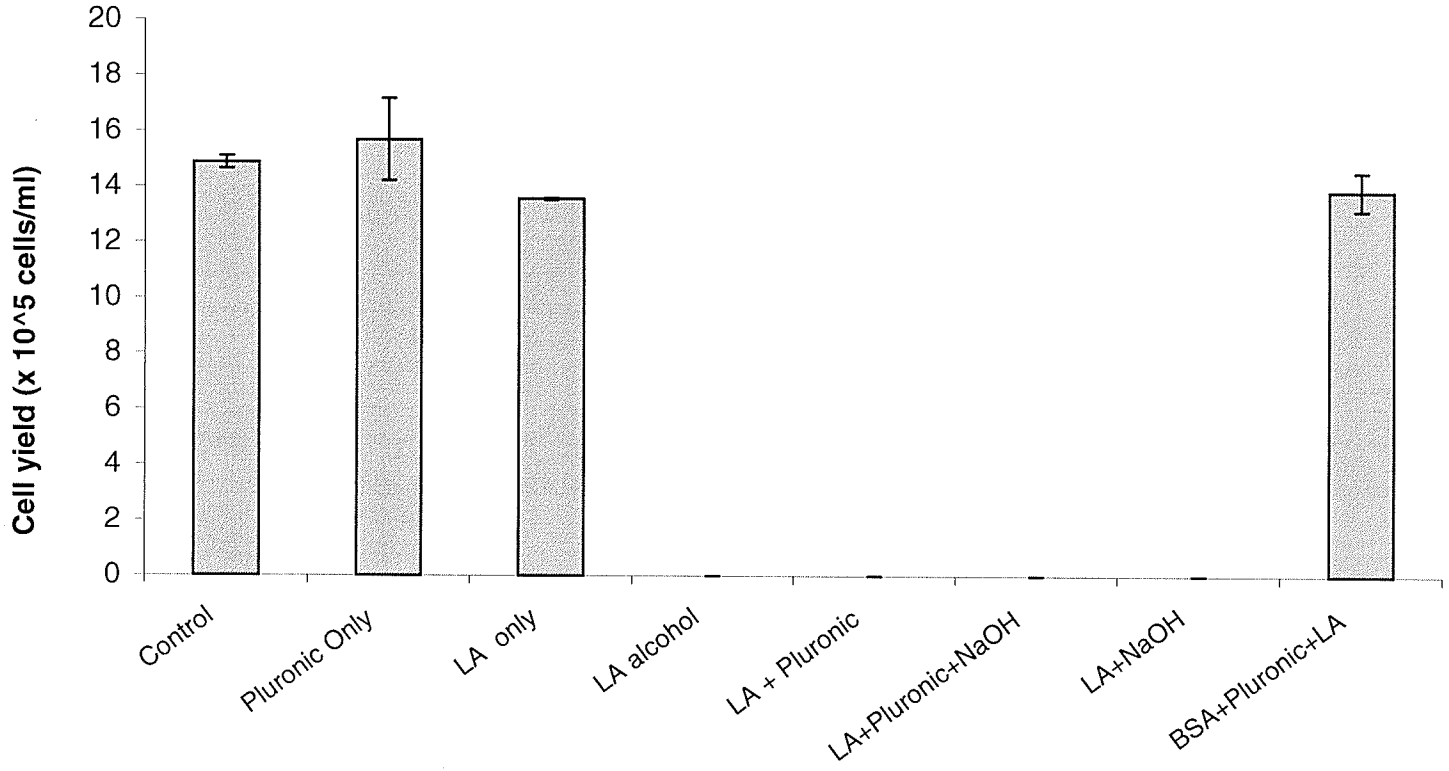


Figure 9.2 Effect of 20 μM of linoleic acid on the growth of CC9C10 cells.

Cells were cultured and linoleic acid stock was prepared as above except for different concentrations. Error bars represent standard deviation, (n=2).

2. Effect of Linoleic acid on the growth of CHO 81 and Hybridoma CC9C10 cells

Linoleic acid is a component of serum that is considered essential for mammalian cell growth. When provided in the culture medium, linoleic acid is incorporated mainly in the cell membrane. Linoleic acid has been shown to increase the growth and product formation of CHO (Schmid 1991 et al.) and hybridoma CC9C10 (Butler 1995) cells. However monoclonal antibody production was suppressed after continued passaging in linoleic acid supplemented medium (Butler et al. 1995). Therefore, before testing any shear protective effect of linoleic acid we studied its effect on growth of CHO 81 and CC9C10 hybridoma cells. This is because any negative effect on growth and product formation would counteract any beneficial protective effect.

Linoleic acid was complexed with BSA and provided in the medium. CHO 81 cells were cultured in either 25-cm^2 T-flasks or 100 mL spinner flasks while hybridoma CC9C10 were cultured in 75 cm^2 T-flasks. Cells were consecutively passaged several times (total number of passages (n) =5, 3 and 10 for CHO 81 in T-flasks, spinner flasks, and hybridoma in T-flasks respectively) in the presence of different linoleic acid concentrations (all complexed with BSA). The control cultures had no linoleic acid but had 0.1 mg/mL BSA. In an earlier experiment (results not shown) the presence of BSA showed no significant effect on the growth of cells.

Linoleic acid affected the growth of mammalian cells (in μM concentrations). Results show that concentrations up to 20 μM of linoleic acid enhanced the growth of CC9C10 hybridoma cells (results not shown). Higher concentrations were inhibitory. Growth

results match previous results in our laboratory (Butler et al. 1995). Similarly but with less effect linoleic acid enhanced the growth of CHO 81 cells in agitated cultures (Figure 9.3). The optimum concentration for growth in spinner flasks was 5 μM of linoleic acid although no extra benefit was achieved for total EPO produced. Although the difference in cell growth data between cells cultured at linoleic acid concentrations of 0, 5 or 10 μM is minimal, T-test indicates a significant difference ($p = 0.01$, 0.02 and < 0.001 for "0 and 5", "5 and 10", and "10 and 20" respectively) and an optimal concentration of 5 μM.

On the other hand, T-flask data for CHO 81 cells showed no significant effect (Figure 9.4). The cell yield of the control was 6.24×10^5 cells/mL with no significant difference in cultures supplemented with linoleic acid. There was no significant effect of linoleic acid on the EPO productivity with mean production of 291 IU/mL for concentrations of up to 10 μM. Higher concentrations were not tested. The cell yield in T-flasks in the control was below that in spinner flasks. This difference may be due to the better mixing in the spinner flasks or to a different batch of cells, which differed in absolute passage number (from time of transfection). Higher cell yields may be obtained at higher passage number as can be seen in Figures 5.1 and 5.2.

3. The protective effect of linoleic acid on mammalian cells:

Previous findings by Butler et al. (1999) showed that linoleic acid significantly protected the cells and decreased the rate of cell destruction at high agitation speeds. In their

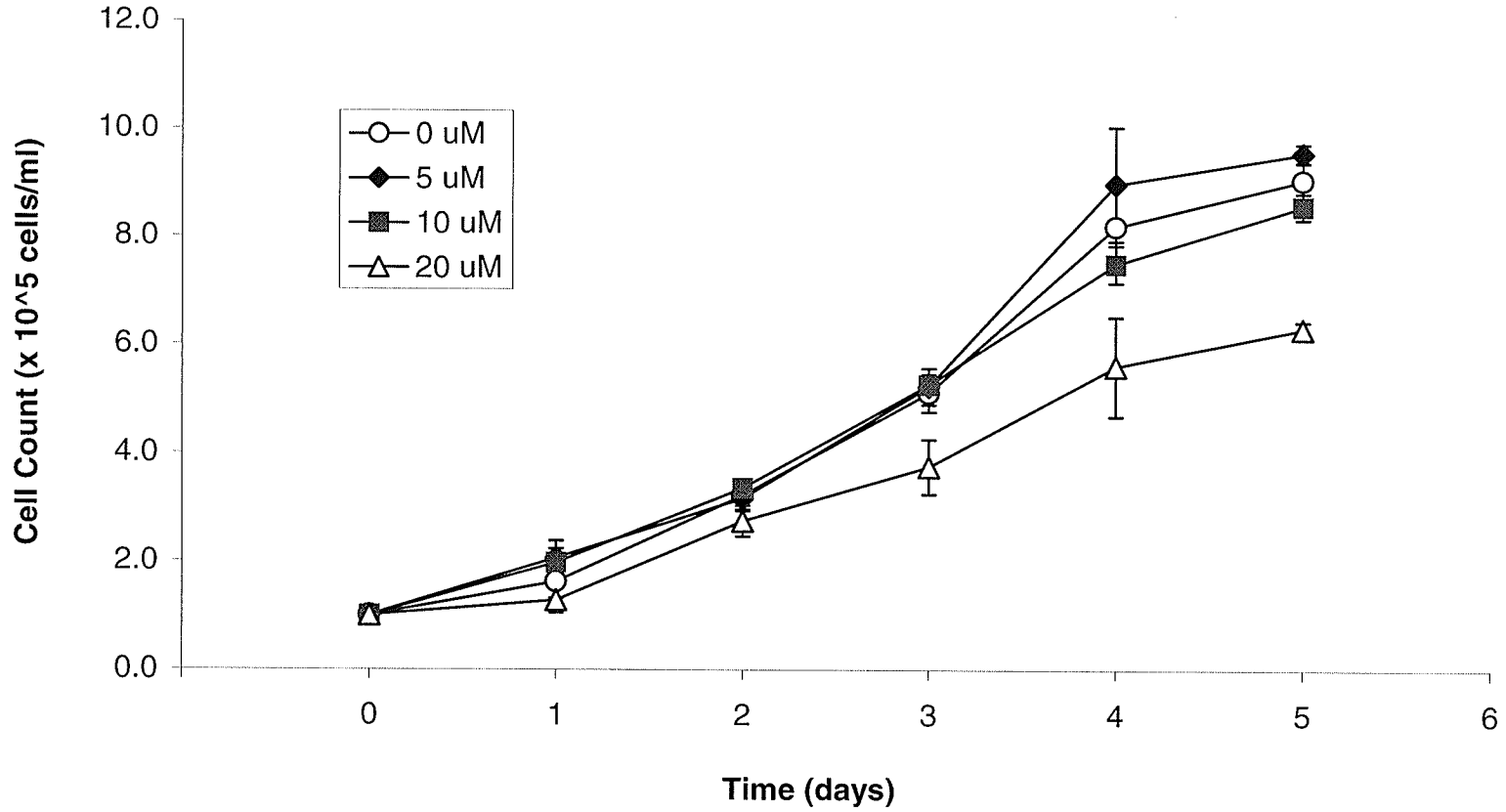


Figure 9.3 Effect of linoleic acid on the growth of CHO 81 cells in agitated cultures.

Cells were cultured in 100 mL spinner flasks for 4 days and agitated at 45 rpm. Linoleic acid was complexed with 0.1mg/mL BSA. Error bars represent standard error of the mean, (n=3).

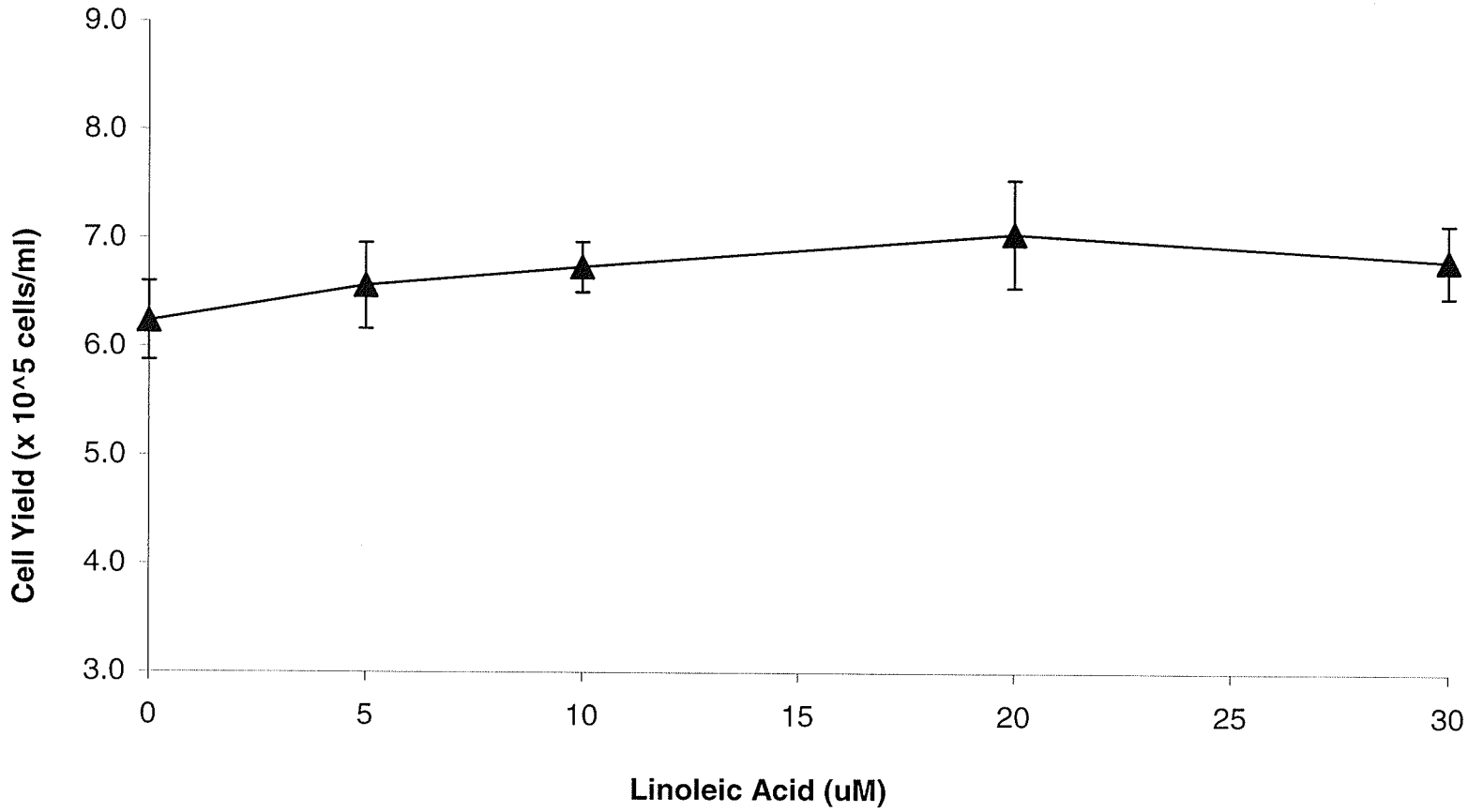


Figure 9.4 Effect of linoleic acid on the growth of CHO 81 cells in stationary T-flasks.

Cells were cultured in 25 cm^2 T-flasks for 4 days. Linoleic acid was complexed with BSA (0.1 mg/mL). Error bars represent standard error of the mean, (n=5).

experiment cells at a concentration of 5 x 10^5 cells/mL were agitated at 470 rpm in 250 mL spinner flasks with a 200 mL culture volume in the absence of Pluronic F68. In this thesis Pluronic F68 was also shown to be protective to cells at a high agitation rate of 500 rpm (Figure 5.4). A comparison is attempted here to study the protective effect of linoleic acid and Pluronic F68 on hybridoma cells.

In sparged cultures Pluronic F-68 protects cells by decreasing the cell to bubble attachment, possibly by decreasing the cell surface hydrophobicity of CC9C10 cells as shown by the decrease in the hydrophobicity index of CHO 81 cells in the presence of Pluronic F68 (Chapter 4). To compare this with the protective effect of linoleic acid we tested the effect of linoleic acid on the hydrophobicity indices of CC9C10 and CHO 81 cells.

Pluronic F68 also increases the cells' resistance to shear in the absence of bubbles as shown by the increase in the survival of cells subjected to high shear rates produced by a viscometer in the presence of Pluronic F68 (Chapter 5). Therefore we also tested the effect of linoleic acid on the sensitivity of CC9C10 hybridoma cells to shear stress in the absence of bubbles using the same viscometer used to test the effect of Pluronic F68.

3.1 The effect of linoleic acid on the hydrophobicity index of CHO and Hybridoma CC9C10 cells.

Using a previously developed method (see chapter 4 for details) to measure the adhesion of cells to hydrophobic surfaces, we measured the hydrophobicity index of CHO 81 cells

and hybridoma CC9C10 cells at different concentrations of linoleic acid. Briefly the method depends on the difference in the distribution of cells between an aqueous and an oil phase. Cells suspended in PBS were vortexed with an equal volume of canola oil. The number of cells in the aqueous phase was determined by haemocytometer counting using trypan blue exclusion dye and subtracted from the total cell number to give the percentage of cells in the hydrophobic layer. Using this method the hydrophobicity index of CC9C10 cells cultured in 175 cm^2 stationary T-flasks and the hydrophobicity index of CHO 81 cells cultured in agitated spinner cultures were determined. The cells were cultured in different concentrations of linoleic acid for at least 5 consecutive passages before measuring the hydrophobicity index of the cells.

Results reveal a concentration dependent and statistically significant increase in the hydrophobicity index of CC9C10 by linoleic acid (Figure 9.5). The hydrophobicity index of both CHO 81 and hybridoma CC9C10 cells increased significantly although it reached a plateau at concentrations higher than 5 μM linoleic acid with CHO 81 cells while it increased continuously from 43 at 0 μM to 67.3 at 50 μM of linoleic acid for CC9C10 cells. The effect on the cell membrane hydrophobicity index correlates with the effect on cell growth for CHO 81 cells. The optimal concentration for cell growth in spinner flasks was 5 μM for CHO 81 cells (Figure 9.3) after which no further increase occurred. The same concentration was the minimum tested concentration to change the hydrophobicity index of CHO 81. On the other hand CC9C10 had an optimal concentration of 20 μM for cells growth but CSH increased continuously up to 50 μM linoleic acid. This may reflect the difference in the cell membrane lipid composition of the two cell lines.

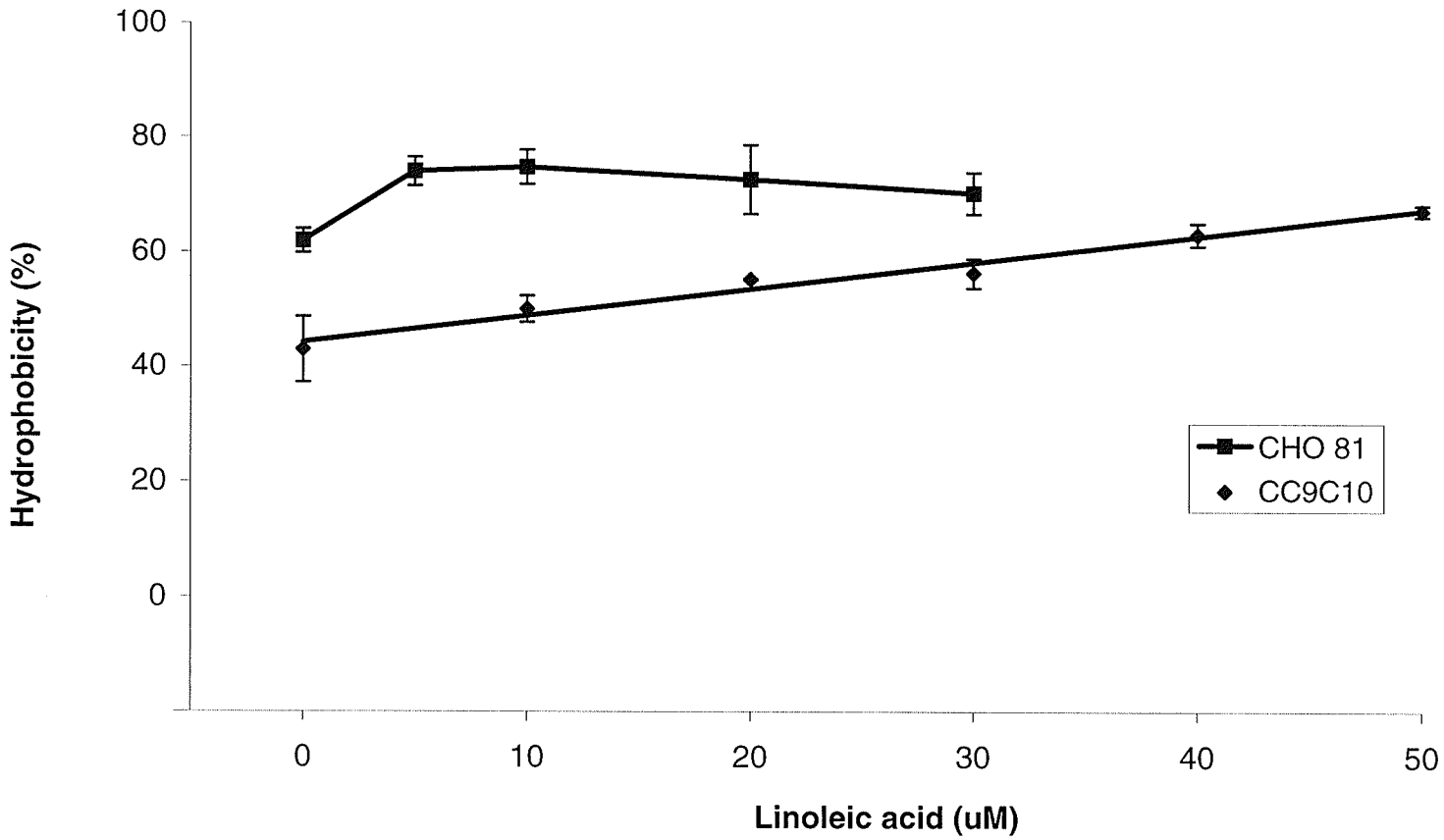


Figure 9.5 Effect of linoleic acid on the hydrophobicity index of CHO 81 and hybridoma CC9C10.

Cells in PBS (2 mL) were mixed with an equal volume of linoleic acid and vortexed for 10 sec. The two phases were let to settle and cells were counted. Error bars represent standard error of the mean, (n=3).

The hydrophobicity index in the absence of linoleic acid was different from the one found in the absence of Pluronic F-68 in Figure 4.2, chapter 4. This difference was not expected since in both cases there was no linoleic acid or Pluronic F68 and there was no difference in the treatment of cells except for the presence of BSA (fatty acid free) in the culturing medium. Hybridoma cells in Figure 9.5 were cultured in the presence of BSA while cells in Figure 4.2, chapter 4 were cultured in the absence of BSA. The difference might be due to the presence of BSA or the difference in the cells' passage number. More work has to be done in this area to reveal the important variable that makes the hydrophobicity change over time.

3.2 Effect of Linoleic acid on cells' resistance to shear in the absence of bubbles:

Using a viscometer that can produce shear rates of up to 12, 000 s^{-1} the protective effect of linoleic acid was tested. Cells supplemented with linoleic acid were tested by taking 0.55 mL of a cell suspension at 37° C and shearing at 12,000 s^{-1} for hybridoma cells. A shear rate of 12,000 s^{-1} was used for CC9C10 cells because these cells showed a higher resistance to shear as discussed previously in chapter 5. Thus high shear rates were used to increase the sensitivity of the measurement by causing a higher amount of cell destruction in a specific time period. The percentage of viable cells remained after shearing from original was compared with control cells that were cultured in un-supplemented growth medium. The concentrations of linoleic acid in CC9C10 cell cultures were 0 to 50 μM because the optimal concentration for cell growth was 20 μM. All cell suspensions contained 0.1 mg/mL BSA during shearing (see below).

Figure 9.6 shows the results for CC9C10 hybridoma cells. There was no significant difference in the shear tolerance of CC9C10 supplemented with different linoleic acid concentrations. After 12 minutes of continuous shearing 60% of the viable cells present before shearing remained. The effect was similar to that reported in Figure 5.5 where the rate was slightly lower at 11,000 s^{-1}. In Figure 5.5 (chapter 5) 50% of viable cells remained after 6 minutes compared with an average of 70% at 12,000 s^{-1} shear rate in figure 9.5 in this chapter. The only difference between the treatments of cells in those experiments was the presence of BSA during both the culture and shearing period.

3.3 Effect of BSA on CHO 81 cells resistance to shear.

BSA is a cell protective agent that has been used frequently in serum-free media. Cells in Figure 9.5 showed a higher shear tolerance than cells in Figure 5.5 in chapter 5. The difference was the presence of BSA. Therefore an experiment was set up here to test the effect of BSA in the absence of bubbles on protecting cells from shear forces.

CC9C10 cells growing in the absence of BSA were sheared with or without BSA at a shear rate of 11,000 s^{-1} (number of trials, n=4). The shearing experiment was set up exactly as previous experiment (Figure 9.6) except for the presence or absence of BSA and the difference of shear rate.

Results in Figure 9.7 show the effect of BSA on the shear sensitivity of CC9C10 cells. There was no significant difference due to the presence of BSA. In both cases around 50% of the original number of viable cells remained after 9 minutes of shearing.

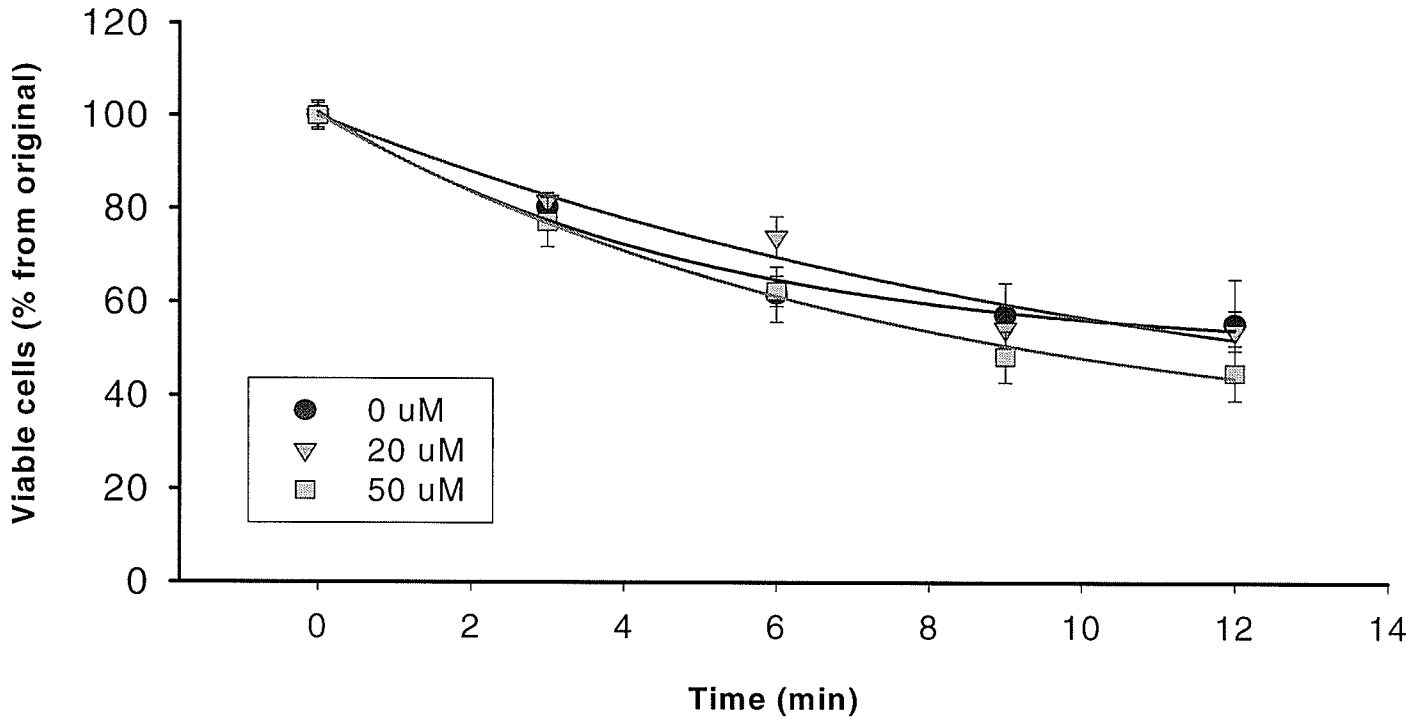


Figure 9.6 The effect of linoleic acid on the shear sensitivity of CC9C10 cells under 12,000 s-1 shear rate.

A sample of cells suspension (0.55 mL) containing 0.1 mg/mL BSA were placed between the cone and plate of Rheolab MC1 viscometer. Cells were subjected to a shear rate of 12,000 s^{-1} at a temperature of 37 °C. Error bars represent standard error of the mean, (n=5).

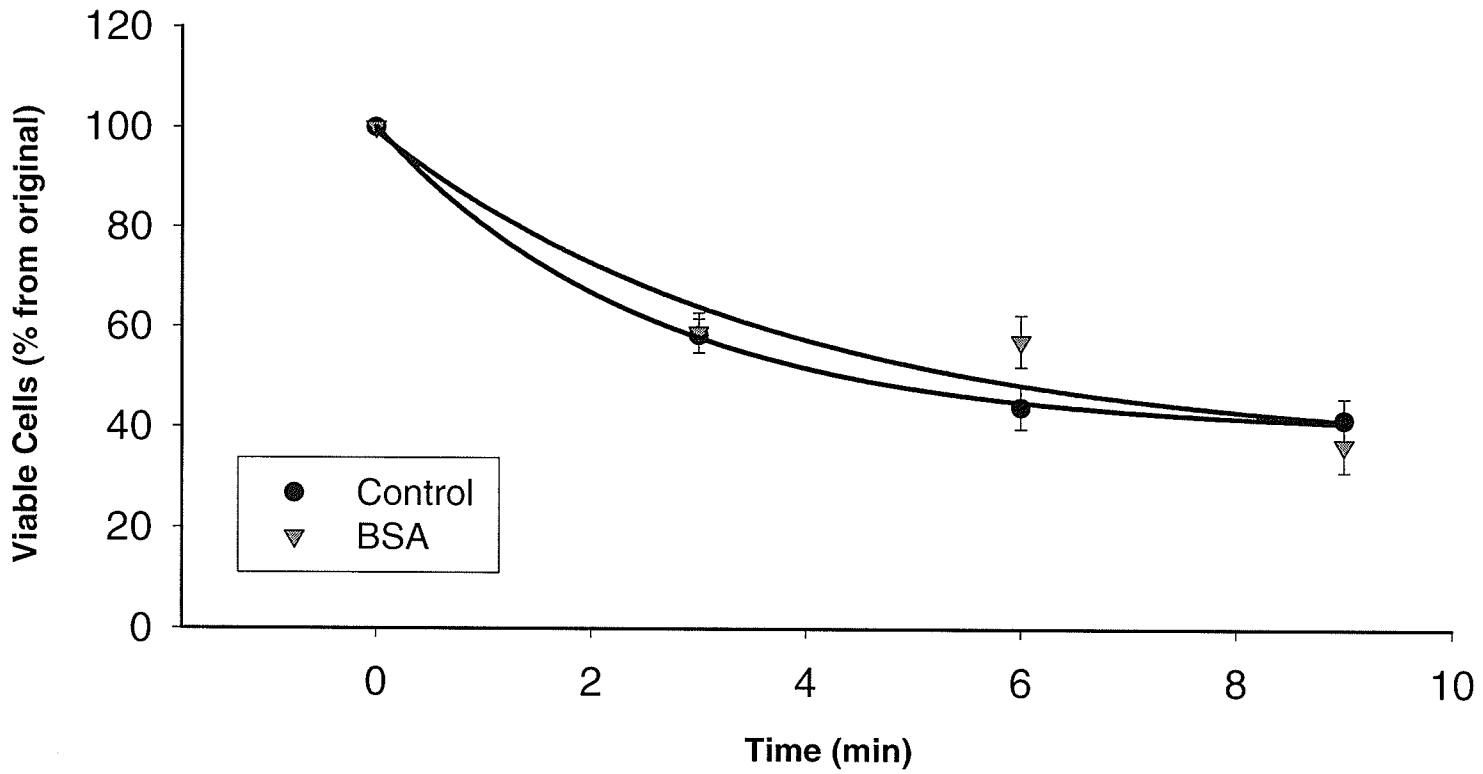


Figure 9.7 Effect of BSA on the sensitivity of CC9C10 cells to shear rates of 11,000 s^{-1}.

Cells were kept at 37 °C in a protein-free and Pluronic-free medium. Sample of 0.55 mL were sheared in a cone and plate viscometer. Viable cells were counted after shearing using the trypan blue exclusion dye method. Error bars represent standard error of the mean, (n=4).

4. The effect of linoleic acid on Plasma membrane fluidity of CC9C10 Hybridoma cells

Since the protective effect of linoleic acid is not shown in the viscometer experiment but still can be shown in spinner flasks experiments we wanted to explore the nature of the protection. Therefore an experiment was set up to show the effect of linoleic acid on the plasma membrane fluidity.

Membrane lipids are in a more or less "fluid" state. Membrane fluidity arises from fast rotations around the long molecular axes of the lipid molecules and from rotational isomerisations around carbon-carbon bonds. Information on the behavior of the lipid molecules can be available from (Electron Spin Resonance) ESR signals of lipid spin labels. Examples of spin labels are 5-doxyl stearic acid. The 5-doxyl stearic acid label probes the membrane bilayer near the surface (Zaleska et al. 1989).

Plasma membrane fluidity was measured by the change in the order parameter in an ESR instrument. The effective order parameter (S eff) determined by E.S.R. spectroscopy is a measure of the amplitude and rate of motion of acyl chains or membrane fluidity (Calder et al. 1994). For more detailed explanation of ESR see chapter 7.

As discussed in more detail in chapter 2, CC9C10 cells were prepared by passaging them for at least 3 passages in linoleic acid supplemented medium at concentrations of 0, 20 and 30 μM. Cells centrifuged and re-suspended in PBS were incubated with a dry film of spin label. Cells were re-suspended in PBS and introduced into a capillary to take the ESR measurements.

ESR results in Table 1 show that the linoleic acid concentration in culture significantly affected the order parameter. The order parameter was significantly lower (P = 0.02) for cells treated with 20 μM linoleic acid compared with the control (No linoleic acid supplementation). The order parameter (average) has decreased from 0.624 to 0.612. A lower order parameter indicates a higher degree of disorder i.e. higher plasma membrane fluidity. The order parameter has increased from 0.612 to 0.624 again significantly (P = 0.01) with 30 μM of linoleic acid indicating a decrease in the membrane fluidity. There was no significant difference between the control (0 μM linoleic acid) and 30 μM linoleic acid.

Table 9.1 The order parameter for CC9C10 cells cultured with different LA concentration.

Conc. of LA	S1	S2	S3	S4	S_{av}*	± SD*
0 μM	0.627	0.630	0.622	0.618	**0.624**	0.0052
20 μM	0.611	0.616	0.603	0.616	**0.612**	0.0063
30 μM	0.614	0.628	0.624	0.631	**0.624**	0.0075

*S_{av} is the average order parameter and SD is the standard deviation.

Discussion:

Linoleic acid uptake by the cells has been shown to follow passive diffusion kinetics (Abumrad 1998) therefore the higher the concentration in the medium the higher the rate of uptake will be. Furthermore the process itself seems to be uncontrolled so that an excess of fatty acids will be accumulated in the cells as lipid droplets (Schneeberger et al.

1971). Results here show that high concentrations of linoleic acid (20 μM here) lead to cell death in the absence of a complexing agent like BSA. It is likely that in the presence of complexing agent most of the linoleic acid molecules are bound and only a small fraction is available to cells. This suggests that an equilibrium happens between complexed linoleic acid, free linoleic acid and cell-bound linoleic acid in the culture. On the other hand, Pluronic F68 was able to complex the linoleic acid and make it pass through the filter during the filter sterilization process but was not able to prevent linoleic acid adverse effect on the cell.

Upon uptake, linoleic acid is incorporated into the cells, increasing the ratio of unsaturated to saturated fatty acids in the cells (Schneeberger et al. 1971). Additions of 15-40 μM of linoleic acid in serum free media have been shown to give the greatest increase in cell numbers, while higher concentrations have shown inhibition (Kovar and Franek 1986). The enhancement is also reversible, thus a constant supply of linoleic acid is required to maintain the enhanced growth rate. The improvement is likely coupled to the cells' requirement for fatty acids for optimal cell growth (Butler, 1995). The exact mechanism for the growth enhancement by linoleic acid addition in hybridoma CC9C10 cells or for the enhancement of cell robustness is yet unknown. It is speculated that because linoleic acid is an essential fatty acid required for maintaining normal membrane structure, its incorporation into the phospholipid membrane optimizes cell membrane fluidity and alters membrane surface characteristics (Butler et al. 1995 and Butler et al. 1997). Butler et al. (1997) dismissed the possibility that the fatty acids are providing extra energy to the cells. The evidence came from measuring the radio-labelled ^{14}C carbon

dioxide after feeding the cells with radio-labelled ^{14}C linoleic acid. Another possibility, that the fatty acids are affecting the eicosanoid composition of the cells, was also ruled out. This is because arachidonic acid did not show any stimulatory effect on cells.

Addition of linoleic acid to serum free media formulations has been shown in this study to cause growth promotion of hybridoma CC9C10 cells (stationary cultures) and CHO 81 cells (agitated cultures). Results here show an optimal concentration of 20 μM of linoleic acid for CC9C10 cells (results not shown), which matches the results previously reported by Butler et al. (1995), and 5 μM for CHO 81 cells (Figure 9.3). There was no direct effect on the recombinant protein production in CHO 81 cells. These effects were evident in agitated cultures but not apparent in T-flask cultures of CHO 81 cells. The reason for a difference in the cells' requirements for unsaturated fatty acids in the two methods of culture for CHO 81 cells is not clear. We speculate that there is a higher availability of linoleic acid to cells in the suspension culture than in adherent cell cultures due to the presence of attachment proteins. Linoleic acid is hydrophobic mainly due to a long alkyl chain and is adsorbed more easily by a hydrophobic membrane. However, it is unclear how this might be related to the mode of culture.

Linoleic acid did not protect hybridoma CC9C10 cells and CHO 81 cells in the same physical manner as Pluronic F68. Opposite to the effect of Pluronic F68 (chapter 4), linoleic acid increased the hydrophobicity index of the cell. Results of the cell hydrophobicity assay show up to a 30% increase in hydrophobicity of CC9C10 cells

grown in linoleic acid supplemented media. This increase in the hydrophobicity suggests a higher tendency to attach to bubbles and therefore higher cell death rate, however linoleic acid addition is still protective to cells. This suggests that the protective features provided by linoleic are likely to be based on a biological mechanism rather than a physical one.

Linoleic acid has previously been shown to incorporate into the polar lipid fraction of hybridoma cells, which includes the plasma membrane (Butler et al. 1997; Calder et al. 1994). The resulting increase in the ratio of unsaturated to saturated fatty acids, and the decrease in content of fatty acids other that linoleic suggest that linoleic acid is both being incorporated and replacing other fatty acids in the membrane (Butler et al. 1995). These membrane alterations are possibly conferring a more optimal fluidity of the membrane, thereby enhancing cell robustness. However, results of the plasma membrane fluidity show an increase in PMF with higher concentrations of linoleic acid. The results were consistent with Calder et al.'s (1994) results showing an increase in the PMF following the addition of 100 μM of linoleic acid to mitogen stimulated lymphocytes. The difference in the effective concentration between Calder et al. (1994) and this thesis is the difference in the time the cells were exposed to the fatty acid. Calder et al. cultured the cells for 64 hours only. In this thesis the cells were cultured through three consecutive 72 hour passages (total of 216 hours). Furthermore the type of cells used here is different from the cells (lymphocytes) used in the Calder et al. (1994) study.

The effect of linoleic acid on the PMF however does not suggest a higher robustness because higher PMF is associated with less robustness according to Ramirez and Mutharasan (1992). They have showed that Pluronic F68, cholesterol and serum, which have proven protective effects, decrease the PMF of cells in culture (Ramirez and Mutharasan (1990). Therefore it is likely that linoleic acid is increasing the robustness of cells in agitated cultures (as shown by Butler et al. 1999) by other means which are not clear.

Viscometer results did not show any protective effect of linoleic acid. Probably this is because the difference in the robustness of the cells with different concentrations of linoleic acid is below the detection limit of the viscometer.

Many functions have been attributed to BSA in serum free media. Several researchers reported a supportive effect for cell growth and product development (Castro et al. 1996). However, the BSA quality is a concern since the stimulatory effects were only observed with fatty acid free BSA (Castro et al. 1996). This is probably due to the negative effect of oxidized fatty acids adsorbed with BSA during the purification process (Jenkins et al. 1994). It is believed that BSA protects from shear stresses, neutralizes toxic waste products, and works as a source of lipids, hormones and trace metals that might be adsorbed to it (Qi et al. 1996; Iscove et al. 1980). BSA has been shown to work as an effective carrier molecule as it does in physiological conditions and to decrease the surface tension of liquids due to its amphipathic nature.

The effect of BSA on the hydrophobicity index of hybridoma cells has been shown in chapter 4. This effect is likely to be responsible (at least partially) for the protective effect of BSA in sparged cultures. However in the absence of bubbles BSA showed no significant protective effect for the same hybridoma cells under high shear forces. This was found at a BSA concentration of 0.1mg/mL. Higher concentrations might show different results. However, higher concentrations of BSA are undesired in serum free formulations because it 1) increases the undefined components in the medium 2) purification of recombinant proteins would be harder with the presence of higher concentrations of BSA. BSA is not expected to dissolve or interact covalently with the cell membrane due to its large molecular size.

Conclusions:

- Linoleic acid is insoluble in water and can be introduced into growth medium by several ways including complexation with BSA, Pluronic F68 or dissolving by alkalinization to convert it into sodium linoleate.
- Linoleic acid is inhibitory to cells in culture. However the inhibition is dependent on the way the fatty acid is introduced into the medium and its concentration.
- Pluronic F68 can complex linoleic acid however it can not ameliorate its inhibitory effect on cells.
- The optimum concentration of linoleic acid was 5 μM for CHO 81 cells in agitated cultures.

- There was no significant effect of linoleic acid at concentrations of up to 30 μM on CHO 81 cells in stationary T-flasks.
- Linoleic acid increases the hydrophobicity index of CHO 81 cells and CC9C10 hybridoma cells. The index increased at concentrations up to 5 μM linoleic acid in CHO 81 cells while it continued to increase up to 50 μM in CC9C10 cells.
- There was no significant effect of linoleic acid on the shear tolerance of CC9C10 supplemented with different linoleic acid concentrations as measured by a cone and plate viscometer.
- BSA showed no significant effect on the shear sensitivity of hybridoma CC9C10 cells under high shear stresses in the absence of bubbles.
- Linoleic acid increased significantly the plasma membrane fluidity of CC9C10 cells at 20 μM.

Chapter 10: Conclusions

What are the main findings of the thesis?

Pluronic F68 protects CHO cells in the presence of bubbles in a dose-dependent manner with a maximum protection at 0.2% concentration. Pluronic F68 decreases the cell to bubble attachment, at least partially, by decreasing the hydrophobicity index of mammalian cells, which is likely to reflect a decrease in the cell surface hydrophobicity (CSH). The decrease in hydrophobicity index by Pluronic F68 was different for CHO cells versus CC9C10 hybridoma cells, which suggests a difference in the interaction of Pluronic F68 with different cell lines. The maximum effect on CC9C10 hybridoma cells was achieved with 0.05% Pluronic F68 concentration while the maximum effect on CHO cells needed 0.2% Pluronic F68 concentration.

Pluronic increases the cells' resistance to shear forces. This effect can be shown in the complete absence of bubbles. Pluronic F68 increases the resistance of both CHO and CC9C10 although these cells have different base line sensitivities to shear forces. The protective effect was clearly dose dependent in hybridoma cells with maximum protection at 0.1% Pluronic F68 concentration. There was no significant difference between the protective effect on CHO cells at 0.05% and 0.1% Pluronic F68 concentration. However, the mechanism of the protective effect is unclear.

Several mechanisms are suggested of how Pluronic F68 molecules interact with cells to decrease the cell to bubble attachment and increase the cell resistance to shear. They include:

- Increase in the medium viscosity.
- Adsorption of Pluronic F68 molecules by hydrophobic/hydrophilic interactions to the cell outer surface membrane leading to a change in the cell surface hydrophobicity.
- An interaction of the Pluronic F68 molecules with molecules of the cell membrane leading to a change in the lipid/protein organization of the cell membrane.
- An interaction of the Pluronic F68 molecules with molecules of the cell membrane leading to a change in the plasma membrane fluidity (PMF).

In this thesis the following conclusions were made:

- Viscosity does not increase significantly with Pluronic F68 at concentrations that are frequently used in cell culture. Therefore it cannot be a mechanism by which Pluronic F68 works.
- Pluronic F68 decreased the rate of cellular uptake of linoleic acid when complexed with cyclodextrin or when introduced as free fatty acid. This might be due to the decrease in the cell surface hydrophobicity of cells by Pluronic F68.
- Pluronic F68 did not decrease significantly the plasma membrane fluidity of CC9C10 hybridoma cells.

- The concentration of Pluronic F68 in the medium does not decrease significantly during culture as measured by an assay developed for this purpose. This shows that the Pluronic F68 adsorbed onto the cell surface must be below the detection limit of this assay. This shows also that only a very small portion of Pluronic F68 molecules adsorb to the cells.

In this thesis the protective effects of Pluronic F68, linoleic acid and bovine serum albumin (BSA) were compared. Results show that:

- Linoleic acid increased the hydrophobicity index of mammalian cells. This was opposite to the effect of Pluronic F68, which decreased it.
- Linoleic acid caused a significant increase in the plasma membrane fluidity of CC9C10, an effect that was not shown by Pluronic F68.
- Linoleic acid showed no protective effect on mammalian cells in the absence of bubbles under high shear forces, in contrast to Pluronic F68.
- Bovine Serum Albumin (BSA) decreases the hydrophobicity index of mammalian cell membranes but not to same extent as Pluronic F68 does.
- Bovine Serum Albumin (BSA) at 0.1 mg/mL has no significant effect on the cell resistance to shear stress in a bubble free environment, in contrast to Pluronic F68.

Were the original aims of the thesis met?

The aims of the thesis were met. In this thesis the effect of Pluronic F68 on the growth of CHO cells and recombinant protein production was studied. The effect of Pluronic F68 in the absence of bubbles as well as in the presence of bubbles was studied. The interactions of Pluronic F68 with the cell membrane were studied. Finally the effect of Pluronic F68 and linoleic acid was compared.

Why are the results important?

Results are important because they determine the optimal concentration of Pluronic that can be used in the presence of bubbles or in the absence of bubbles. This suggests that Pluronic F68 concentration and its use can be "tailored" to the cell type, the method of culture and bioreactor geometry. The results show some of the effects of Pluronic on the cell membrane characteristics like cell-surface hydrophobicity, strength, and some nutrients uptake. They also suggest mechanisms on how Pluronic might interact with the cell membrane. Finally, the results compare the effects of Pluronic F68 and BSA or linoleic acid.

Although Pluronic F68 is protective for cells in culture, it has some disadvantages:

- It inhibits the growth of some cells in culture as shown at the 0.05% Pluronic F68 concentration for TB/C3 (Al-Rubeai et al. 1992).
- It makes the purification of some recombinant proteins more difficult especially if the molecular weight of the recombinant protein is small and a 10 k Da or smaller filter is used (Pluronic F68 molecular weight is 8.4 k Da).

This will help in searching for a better alternative for Pluronic F68 that will produce the same protective effect but with fewer side effects. It will also establish the minimum quantities required.

How the results might affect bioreactors set up in future?

The use of Pluronic F68 may depend upon the cell line used, and culture vessel geometry. There is some evidence in this thesis that the protective effect of Pluronic F68 on mammalian cells might be different in the different cell lines.

How can this work be continued?

1. Test the protective effect of different concentrations of Pluronic F68 on hybridomas CC9C10 in spinner flasks agitated at 500 rpm where bubble entrainment occurs. This will show if there is a correlation between the effect of Pluronic F68 on the hydrophobicity index of cells and the protective effect in sparged culture.
2. Measure PMF with cells cultured in the presence of Pluronic F68. This can be achieved by growing hybridoma CC9C10 or CHO cells in Pluronic F68 containing medium for several passages and then measuring the PMF. The results can be compared with cells cultured in the absence of Pluronic F68 in the culture medium.
3. Using the hydrophobicity assay, test the effect of different concentrations of BSA on the hydrophobicity index of cells. The protective effect of different

concentrations of BSA can be also tested at high shear rates in the absence of bubbles using the viscometer.

4. Study the effect of trypsin on the shear sensitivity of cells. This can be done by testing the shear sensitivity of CHO cells after varied periods of trypsinization ranging from 30 sec up to 3 min.

References

Absolom, D. R., (1988) The role of bacterial hydrophobicity in infection: bacterial adhesion and phagocytic ingestion. *Canadian Journal of Microbiology*. **34**, 287-298.

Abumrad, N., Harmon, C., Ibrahimi, A. (1998) Membrane transport of long-chain fatty acids: evidence for a facilitated process. *Journal of Lipid Research* **39**: 2309-2318.

Abumrad, N., Coburn, C., Ibrahimi, A. (1999) Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochimica et Biophysica Acta* **1441**: 4-13.

Al-Rubeai, M., Emery, A. N., Chalder, E. S., Goldman, M. H. (1993) A flow cytometric study of hydrodynamic damage to mammalian cells. *Journal of Biotechnology* **31**: 161-177.

Al-Rubeai, M., Emery, A.N. Chalder, S. (1992) The effect of Pluronic F-68 on hybridoma cells in continuous culture. *Applied Microbiology and Biotechnology* **37**: 44-45.

Al-Rubeai, M. Oh, S. K. W. Musaheb, R., Emery, N. A. (1990) Modified Cellular Metabolism in Hybridomas Subjected to Hydrodynamic and other stresses. *Biotechnology Letters* **12**: 323-328.

Al-Rubeai, M., Singh, R. P., Emery, A. N., Zhang, Z. (1995) Cell Cycle and Cell Size Dependence of Susceptibility to Hydrodynamic Forces. *Biotechnology and Bioengineering* **46**: 88-92.

American Type Culture Collection Catalogue (1990)

BASF Performance Chemicals. Pluronic and Tetronic Surfactants. (1996) BASF corporation

Bassetti, L., Hagendoorn, M., Tramper, J. (1995) Surfactant-induced non-lethal release of anthraquinone from suspension of Morinda citrifolia. *Journal of Biotechnology* **39**: 149 - 155.

Bentley, P. K., Gates, R. M. C., Lowe, K. C. de Pomerai, D. I. Walker, J. A. L. (1989) In vitro Cellular Responses to a non-ionic surfactant Pluronic F-68. *Biotechnology Letters* **11**: 111-114.

Biblia, A., Robinson, K. (1995) In pursuit of the Optimal Fed-Batch Process for Monoclonal Antibody Production. *Biotechnology Progress* **11**: 1-13.

Bjare, U. (1992) Serum-Free Cell Culture. *Pharmacology and Therapeutics* **53**: 355-374.

Boyar, S., Guin, K., Kelley, R., Mausner, M. et al. (1977) Analytical Method for Nonionic Surfactants in Laboratory Biodegradation and Environmental Studies. *Enviromental Science & Technology* **11**: 1167-1170.

Butler, M. (1990) Mammalian cell Biotechnology. Oxford University Press. Oxford, UK.

Butler, M,. Huzel, N. (1995) The effect of fatty acids on hybridoma cell growth and antibody productivity in serum-free cultures. *Journal of Biotechnology* **39**: 165-173.

Butler, M., Huzel, N., Barnabe, N. (1997) Unsaturated fatty acids enhance cell yields and perturb the energy metabolism of an antibody-secreting hybridoma. *Biochemical Journal* **322**: 615-623.

Butler, M. Huzel, N. Barnabe, N. Gray, T. Bajno, L. (1999) Linoleic acid improves the robustness of cells in agitated cultures. *Cytotechnology*

Calder, P., Yaqoob, P., Harvey, D., Watts, A., Newsholme, E. (1994) Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. *Biochemical Journal* **300**: 509-518.

Castro, P.M.L., Ison, A. P., Hayter, P. M. and Bull, A. T. (1995) The machroheterogeneity of recombinant human interferon-γ produced by Chinese-hamster ovary cells is affected by the protein and lipid content of the culture medium. *Biotechnol. Appl. Biochem.* **21**: 87-100.

Castro, P. M. L., Ison, A. P., Hayter, P. M., Bull, A. T. (1996) CHO cell growth and recombinant interferon-γ production: Effects of BSA, Pluronic and lipids. *Cytotechnology* **19**: 27-36.

Cherry, R. S. Papoutsakis, E. T. (1986) Hydrodynamic effects on cells in agitated tissue culture reactors. *Bioprocess Engineering* **1**: 29-41.

Cherry, R. S. Hulle, C. T. (1992) Cell death in the thin films of bursting bubbles. *Biotechnology Progress* **8**: 11-18.

Chisti, Y. (1999) Shear sensitivity In: Flickinger, M. C. and Drew, S. W. Encyclopedia of Bioprocess Technology: Fermentation, *Biocatalysis, and Bioseparation* **5**: 2379-2406. New York

Cross, J. Ed. (1987) Non-ionic Surfactants: Chemical Analysis. Marcel Dekker Inc. New York.

Curatola, G., Mazzanti, L., Ferretti, G., Donato, R. (1985) S-100 Protein-Induced Charges in the Physical State of Synaptosomal Particulate Fractions as Monitored by Spin Labels. *Archives of Biochemistry* **240**: 435-445.

Croughan M. S., Hamel, J. P., Wang, D. I. C. (1988) Effects of Microcarrier Concentration in Animal Cell Culture. *Biotechnology and Bioengineering* **32**: 975-982.

Detrait, E., Lhoest, J. B., Bertrand, P., Bosch de Aguilar, Ph. (1999) Fibronectin-Pluronic Co-adsorption on a polystyrene surface with increasing hydrophobicity: relationship to cell adhesion. *J. Biomed Mat. Res.* **45**: 404-413

Dey, D., Boulton-Stone, J. M., Emery, A. N. Blake, J. R. (1997) Experimental comparisons with a numerical model of surfactant effects on the burst of a single bubble. *Chemical Engineering Science* **52**: 2769-2783.

Dorner, A., Wasley, L., Kaufman, R. (1989) Increased Synthesis of Secreted Proteins Induces Expression of Glucose-regulated Proteins in Butyrate-treated Chinese Hamster Ovary Cells. *The Journal of Biological Chemistry* **264**: 20602-20607.

Ener, B., and Douglas, J., (1992) Correlation between cell-surface hydrophobicity of Candida albicans and adhesion to buccal epithelial cells. *FEMS Microbiol. Lett.* **99**, 37-42.

Ezra, R., Benita, S., Ginsburg, I. Kohen, R. (1996) Prevention of oxidative damage in fibroblast cell cultures and rat skin by positively-charged submicron emulsion of alpha-tocopherol. *European Journal of Pharmaceutics and Biopharmaceutics* **42**: 291-298.

Foucher, C., Lagrost, L., Maupoil, V., Le Meste, M., Rochette, L., Gambert, P. (1996) Alterations of lipoprotein fluidity by non-esterified fatty acids known to affect cholesteryl ester transfer protein activity An electron spin resonance study *European Journal of Biochemistry* **236**: 436-443.

Frangos, J. A., McIntire, L. V., Eskin, S. G. (1988) Shear Stress Induced Stimulation of Mammalian Cell Metabolism. *Biotechnology and Bioengineering* **32**: 1053-1060.

Freshney, T. (1992) Animal cell culture. Oxford University Press. Oxford. U.K.

Ghebeh, H., Handa-Corrigan, A., Butler, M. (1998) Development of an assay for the measurement of the surfactant Pluronic F-68 in mammalian cell culture medium. *Analytical Biochemistry* **262**: 39-44

Ghebeh, H., Gillis, J., Butler, M. (2002)[a] Measurement of hydrophobic interactions of mammalian cells grown in culture. *Journal of Biotechnology* **95**: 39-48.

Ghebeh, H., Butler, M. (2002)[b] The protective effect of Pluronic F68 on mammalian cells in a bubble free environment. Submitted for Publication.

Gilbert, R., Nagano, Y., Yokota, T., Hwan, S., Fletcher, T. Fletcher, T. (1996) Effect of lipids on insect cell growth and expression of recombinant proteins in serum-free medium *Cytotechnology* **22**: 211-216.

Goldblum, S., Bae, Y., Hink, F., Chalmers, J., (1990) Protective Effect of Methylcellulose and Other Polymers on Insect Cells Subjected to Laminar Shear Stress. Biotechnology Progress **6**: 383-390.

Goodwin, R. H. (1990) Lipid cell culture supplements. *Nature* **347**: 209-210.

Gor, D. (1993) Large scale animal cell culture. InVitro Cultivation of Animal Cells Butterworth-Heinemann. oxford.

Gorfien, S., Paul, B., Walowitz, J., Keem, R., Biddle, W., Jayme, D. (2000) Growth of NS0 Cells in Protein-Free, Chemically Defined Medium. *Biotechnology Progress* **16**: 682-687.

Gottesman, M. M. (1985) Molecular Cell Genetics John Wiley and Sons New York.

Greff, R.A., Setzkorn, A. E., and Leslie, W. D. (1965) A Colorimetric Method for Determination of parts/million of Nonionic surfactants *Journal of American Oil Chemical Society* **42**, 180-185.

Hamilton, J., Johnson, A., Corkey, B., Kamp, F., (2001) Fatty Acid Transport-The diffusion mechanism in model and biological membranes. Journal of Molecular Neuroscience **16**: 99-108.

Handa-Corrigan, A., Emery, A. N. Spier, R. E. (1989) Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubble. *Enzyme Microbe Technology* **11**: 230-235.

Handa-Corrigan, A. Weeratunge, N. P. Braybrook, J. H. et al. (1997) In-vitro Toxicological Effects of Pluronic F-68. *ESACT* :121-126.

Handa-Corrigan, A. Zhang, Z., Brydges, R. (1992) Surface Active Agents in Animal Cell Cultures In: Animal Cell Biotechnology Vol 5 Academic Press

Helenius, A., Simons, K., (1975) Solubilization of membrane by detergents *Biochimica et Biophysica Acta* **415**: 29-79.

Hink, F. (1991) A serum-free medium for the culture of insect cells and production of recombinant proteins. *In Vitro Cellular and Developmental Biology* **27A**: 397-401.

Hoseney, R. C., Finney, K. F., Shogren, M. D. (1972) Functional (Breadmaking) and Biochemical Properties of Wheat Flour Components. IX. Replacing Total Free Lipid with Synthetic Lipid. *Cereal Chemistry* **49**: 366-371.

Ingram, D.A., Forman, M.B., Murray, J.J. (1993) Activation of complement by fluosol attributable to the Pluronic detergent micelle structure. *Journal of Cardiovascular Pharmacology* **22**: 456-461.

Iscove, N., Guilbert, L., Weyman, C., (1980) Complete Replacement of Serum in Primary Cultures of Erythropoietin-Dependent Red Cell Precursors (CFU-E) By Albumin, Transferrin, Iron, Unsaturated Fatty Acid, Lecithin and Cholesterol. *Experimental Cell Research* **126**: 121-126.

Jenkins N., Castro, P., Menon, S. Ison, A. Bull, A. (1994) Effect of lipid supplements on the production and glycosylation of recombinant interferon-γ expressed in CHO cells. *Cytotechnology* **15**: 209-215.

Jewell, R. C., Khor, S. P. Kisor, D. F. LaCroix, K. A. K. et al. (1997) Pharmacokinetics of RheothRx Injection in Healthy Male Volunteers. *Journal of Pharmaceutical Sciences* **86**: 810-812.

Jordan, M., Sucker, H., Einsele, A., Widmer., Eppenberger, H. M. (1994) Interactions between Animal Cells and Gas Bubbles: The Influence of Serum and Pluronic F68 on the Physical Properties of the Bubble Surface. *Biotechnology and Bioengineering* **43**: 446-454.

Khang, G., Jeong, B. J., Lee, H. B. and Park J. B., (1995) Biocompatibility of Polysulfone II. Platelet adhesion and CHO cell growth. *Bio-Medical Materials and Engineering.* **5**: 259-273.

Keefe, J. H., Grines, C. L., DeWood, Schaer, G. L. et al. (1996) Poloxamer 188 as an Adjunct to Primary Percutaneous Transluminal Coronary Angioplasty for Acute Myocardial Infarction. *American Journal of Cardiology* **78**: 747-750).

Keen, M. J., Rapson, N. T. (1995) Development of a serum-free culture medium for the large scale production of recombinant protein from a Chinese hamster ovary cell line. *Cytotechnology* **17**: 153-163.

Kier, L. D., Wagner, L. M. Wilson, T. V. Short, R. D. et al. (1995) Cytotoxicity Of Ethylene Oxide/Propolyene Oxide Cpolymers In Cultured Mammalian cells. *Drug and Chemical Toxicology* **18**: 29-41.

King, A., Davey, M., Mellor, I., Mulligan, B., Lowe, K., (1991) Surfactant effects on yeast cells. *Enzyme and Microbial Technology* **13**:148-153.

Kjelleberg, S., Largercrantz, C., and Larsson, TH.,. (1980) Quantitative analysis of bacterial hydrophobicity studied by the binding of dodecanoic acid. *FEMS Microbiol. Lett.* **7**: 41-44.

Kleinfeld, A. (2000) Lipid Phase Fatty Acid Flip-Flop, Is It Fast Enough for Cellular Transport. *Journal of Membrane Biology* **175**: 79-86.

Kretzmer, G., Schugerl, K. (1991) Response of mammalian cells to shear stress. *Applied Microbiology and Biotechnology* **34**: 613-616.

Krevelen, D. (1972) Typology of polymers In: Properties of Polymers Correlation with Chemical Sturcture. Elsevier Publishing Company. Amsterdam.

Kunas, K. T., Papoutsakis, E. T. (1990) Damage Mechanisms of Suspended Animal Cells in Agitated Bioreactors with and without Bubble Entrainment. *Biotechnology and Bioengineering*; **36**: 476-483.

Lakhotia, S., Papoutsakis, E. T. (1992) Agitation Induced Cell Injury in Microcarrier Cultures. Protective Effect of Viscosity Is Agitation Intensity Dependent: Experiments and Modeling. *Biotechnology and Bioengineering* **39**: 95-107.

Lakhotia, S., Bauer, K. D. Papoutsakis, E. T. (1992) Damaging Agitation Intensities Increase DNA synthesis Rate and Alter Cell-Cycle Phase Distributions of CHO cells. *Biotechnology and Bioengineering* **40**: 978-990

Lakhotia, S., Bauer, K. D., Papoutsakis, E. T. (1993) Fluid-Mechanical Forces in Agitated Bioreactors Reduce the CD13 and CD33 Surface Protein Content of HL60 Cells. *Biotechnology and Bioengineering* **41**: 868-877.

Lane, T.A., Lamkin, G. E. (1984) Paralysis of phagocyte migration due to an artificial blood substitute. *Blood* **64**: 400-405.

Lane, T. A., Lamkin, G.E. (1986) Increased infection mortality and decreased neutrophil migration due to a component of an artificial blood substitute. *Blood* **68**: 351-354.

Lao M. S., Schalla C. (1996) Development of a serum-free medium using computer-assisted factorial design and analysis *Cytotechnology* **22**: 25-31.

Li, J., Caldwell, K. D., Rapoport, N. (1994) Surface Properties of Pluronic-Coated Polymeric Colloids. *Langmuir* **10**: 4475-4482.

Li, J. Caldwell, K. D. (1996) Plasma protein interactions with Pluronic F68 treated colloids. Colloids and Surfaces B: *Biointerfaces* **7**: 9-22.

Lin, W. Higgins, D. Pacheco, J. Aletta, J. et al. (1993) Manganese Induces Spreading and Process Outgrowth in Rat Pheochromocytoma (PC12) Cells. *Journal of Neuroscience Research* **34**: 546-561.

Ludwig, A. Kretzmer, Schugerl, K. (1992) Determination of a 'Critical shear stress level' applied to adherent mammalian cells. *Enzyme and Microbial Technol*ogy **14**: 209-213.

Ludwig, A. Kretzmer, G. (1993) Shear stress induced variation of cell condition and productivity. *Journal of Biotechnology*; **27**: 217-223.

MacIntyre, F. (1968) *Journal of Physical Chemsitry* **72**: 589-592.

Mackinnon, W. B., May G, L. Mountford, C. E. (1992) Esterified Cholesterol and Triglyceride are present in plasma membranes of Chinese hamster ovary cells. *European Journal of Biochemistry* **205**: 827-839.

Magnusson, K., and Johansson, G., (1977) Probing the surface of Salmonella typhimurium and Salmonella minnesota sr and r bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charged polymers. *FEMS Microbiol. Lett.* **2**, 225-228

Marquis, C., Low, K., Barford, J., Harbour, C., (1989) Agitation and aeration effects in suspension mammalian cell cultures. *Cytotechnology* **2**, 163-170.

Martens, D. E., de Gooijer, C. D., Beuvery, E. C., Tramper, J. (1992) Effect of Serum Concentration on Hybridoma Viable Cell Density and Production of Monoclonal Antibodies in CSTR and on Shear Sensitivity in Air-Lift Loop Reactors. *Biotechnology and Bioengineering* **39**: 891-897.

McQueen, A., Bailey, J. (1989) Influence of Serum Level, Cell line, Flow Type and Viscosity on Flow-Induced Lysis of Suspended Mammalian Cells. *Biotechnology Letters* **8**: 531-536

Meier, S.J., Hatton, T.A., and Wang, D.I.C., (1999) Cell Death from Bursting Bubbles: Role of Cell Attachment to Rising Bubbles in Sparged Reactors. *Biotechnology and Bioengineering* **62**, 468-478.

Messi, F. (1991) Tuning of structure and function of Chinese hamster ovary (CHO) cells by systematic design of defined micro-environment. Doctoral thesis, Swiss Federal Institute of Technology.

Michaels, J. D., Peterson, J. F., McIntire, L. V., and Papoutsakis, E. T. (1991) Protection Mechanisms of Freely Suspended Animal Cells (CRL 8018) from Fluid-Mechanical Injury. Viscometric and Bioreactor Studies Using Serum, Pluronic F-68 and Polyethylene Glycol. *Biotechnology and Bioengineering* **38**: 169-180.

Michaels, J. D., Nowak, J. E., Mallik, A. K., Koczo, K. et al. (1995) Analysis of Cell-to-Bubble Attachment in Sparged Bioreactors in the Presence of Cell-Protecting Additives. *Biotechnology and Bioengineering*; **47**: 407-419.

Michaels, J. D., Mallik, A. K., Papoutsakis, E. T. (1996) Sparging and Agitation-Induced Injury of Cultured Animal Cells: Do Cell-to-Bubble Interactions in the Bulk Liquid Injure Cells? *Biotechnology and Bioengineering* **51**: 399-409.

Miller, D. W., Batrakova, E. V., Waltner, T. O., Alakhov, V. Y. et al. (1997) Interactions of Pluronic Block Copolymers with Brain Microvessel Endothelial Cells: Evidence of Two Potential Pathways for Drug Absorption. *Bioconjugate Chemistry* **8**: 649-657.

Mimura, Y., Lund, J., Church, S., Dong, S. et al. (2001) Butyrate increases production of human chimeric IgG in CHO-K1 cells whilst maintaining function and glycoform profile. *Journal of Immunological Methods* **247**: 205-216.

Mizrahi A (1975) Pluronic polyols in human lymphocyte cell line cultures. *Journal of Clinical Microbiology* **2**: 11-13.

Moghimi, S. M., Hunter, A. C. (2000) Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. *Trends in Biotechnology* **18**: 412-420.

Morgan, J.S., Darling, D.C. (1993) Animal Cell Culture. Bios Scientific Publishers. Oxford

Morrison R., and Boyd R., (1992) Organic Chemistry. Sixth edition. Prentice Hall, New Jersey.

Murhammer, D., Goochee, C. (1990)[a] Structural Features of Nonionic Polyglycol Polymer Molecules Responsible for the Protective Effect in Sparged Animal Cell Bioreactors *Biotechnology Progress* **6**: 142-148.

Murhammer, D., Goochee, C. (1990)b Sparged Animal Cell Bioreactorss: Mechanism of Cell Damage and Pluronic F-68 Protection. *Biotechnology Progress* **6**: 391-397.

Murota, K., Matsui, N., Kawada, T., Takahashi, N., et al. (2001) Influence of fatty alcohol and other fatty acid derivatives on fatty acid uptake into rat intestinal epithelial cells. *Lipids* **36**: 21-26.

Neutra, R., Levi B., Shoham, Y. (1992) Optimization of protein-production by the baculovirus expression vector system in shake flasks. *Applied Microbiology and Biotechnology* **37**: 74-78.

Nie, Z. (1992) Fetuin: its enigmatic property of growth promotion. *American Journal of Physiology* **263**: C551-C562.

O'Connor, K., Papoutsakis, E. (1992) Agitation effects on microcarrier and suspension CHO cells. *Biotechnology Techniques* **6**: 323-328.

Oh, S. K., W. Nienow, A. W. Al-Rubeai, M. and Emery, A. N. (1992) Further studies of the culture of mouse hybridomas in an agitated bioreactor with and without continous sparing. *Journal of Biotechnology* **22**: 245-270.

Papahadjopoulos, D., Cowden, M., Kimeberg, H. (1973) "Role of Cholesterol in Membrane Effects on Phospholipid-Protein Interactions. Membrane Permeability and Enzymatic Activity" *Biochimica et Biophysica Acta* **330**: 8-26.

Papoutsakis, E. (1991) Fluid-mechanical damage of animal cells in bioreactors. *TIBTECH* **9**: 427-437.

Pauling, L., (1967) The Chemical Bond. Cornell University Press, Ithaca, New York.

Philips, H. J., (1973) Dye exclusion tests for cell viability. In: Kruse, PF, and Patterson MK, (eds), Tissue culture: Methods and Applications. (pp 406-408), Academic Press, N.Y.

Pinkerton, F. D., Kirkpatrick, N. D., and Schroepfer, G. J. (1988) Inhibitors of Sterol Synthesis. Exogenous oleate reduces the inhibitory effect of 5α-Cholest-8(14)-EN-3β-OL-15-ONE on the growth of CHO-K1 Cells. *Biochemical and Biophysical Research Communications* **156**: 289-294.

Puck, T., Cieciura, S., T., Robinson, A., (1958) Genetics of Somatic Mammalian Cells. *Journal of Experimental Medicine* **108**: 945.

Qi, Y. M., Greenfield, P. F., Reid, S., (1996) Evaluation of a simple protein free medium that supports high levels of monoclonal antibody production. *Cytotechnology* **21**: 95-109.

Ramirez, O. T., Mutharasan, R. (1990) The Role of Plasma Membrane Fluidity on the shear Sensitivity of Hybridoma Grown under Hydrodynamic Stress. *Biotechnology and Bioengineering* **36**: 911-920

Ramirez, O. T., Mutharasan, R. (1992) Effect of Serum on the Plasma Membrane Fluidity of Hybridomas: An Insight into Its Shear Protective Mechanism. *Biotechnology Progress* **8**: 40-50.

Renner, W. A., Jordan, M., Eppenberger, H. M., (1993) Cell-Cell Adhesion and Aggregation: Influence on the Growth Behavior of CHO Cells. *Biotechnology and Bioengineering* **41**: 188-193.

Rintoul, D. A. Sklar, L. A. and Simoni, R. D., (1978) Membrane Lipid Modification of Chinese Hamster Ovary Cells. *The Journal of Biological Chemistry* **253**: 7447-7452.

Rosenberg, M., Gutnick, D., and Rosenburg, E., (1980) Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Letters* **9**: 29-33..

Sato, J. D. Cao, H. Kayada, Y. Cabot, M. C. Sato, G. H. Okamoto, T. Welsh, C. J. (1988) Effects of proximate cholesterol precursors and steroid hormones on mouse myeloma growth in serum-free medium. *In vitro Cellular and Developmental Biology* **24**: 1223-1228.

Sato, K. Saito, H. Katsuki, H., (1993) Synergism of tocopherol and ascorbate on the survival of cultured brain neurons. *Neuroreport* **4**: 1179-1182.

Savonniere, S., Zeghari, N., Miccoli, L. Muller, S., Maugras, M., Donner, M., (1996) Effects of lipid supplementation of culture media on cell growth, antibody production, membrane structure and dynamics in two hybridomas. *Journal of Biotechnology* **48**: 161-173..

Schaer, G. L., Spaccavento, L. J., Browne, K. F. Krueger, K. A. (1996) Beneficial Effects of RheothRx Injection in Patients Receiving Thrombolytic Therapy for Acute Mycocardial Infarction. *Circulation* **94**: 298-307.

Schmid, G., Zilg, H., Uberhard, U., Johannsen, R. (1991) Effect of free fatty acids and phospholipids on growth of and product formation by recombinant baby hamster kidney (rBHK) and Chinese hamster ovary (rCHO) cells in culture. *Journal of Biotechnology* **17**: 155-167.

Shiragami, N. (1997) Effect of shear rate on hybridoma cell metabolism. *Bioprocess Engineering* **16**: 345-347.

Smith, C. Greenfield, P., Randerson, D. (1987) Shear sensitivity of Cultured Hybridoma Cells (CRL-8018) Depends on Mode of Growth, Culture Age and Metabolic Concentration. *Biotechnology Techniques* **1**: 39-44.

Smith II, C.M., Hebbel, R.P., Tukey, D.P. Clawson, C.C. White, J.G. Vercellotti, G.M. (1987) Pluronic F-68 reduces the endothelial adherence and improves the rheology of liganded sickle erythrocytes. *Blood* **69**: 1631-1636.

Smyth, C.J., Jonsson, P., Olsson, E., Soderlind, O., Rosengren, J., Hjerten, S., and Wadstrom, J., (1978) Differences in hydrophobic surface characteristics of porcine enteropathogeni Escherichia coli with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infect. Immun.* **22**: 462-472.

Spier, R.E. Griffiths, J.B. (1985) *Animal Cell Biotechnology* (Volume 1). Academic press. London. U.K.

Stryer, L., (1988) Biochemistry Freedman and company. New York.

Takahashi, Y., Tadokoro, H., (1973) Structural Studies of Polyethers, $(-(CH_2)_m-O-)_n.X$. Crystal Structure of Poly(ethylene oxide). *Macromolecules* **6** : 672-675.

Tercyak, A.M., Felker, T.E. (1990) Colorimetric assay for pluronic F-68 as measured in isolated rat liver perfusion systems. *Analytical Biochemistry* **187**: 54-55.

Tramper, J., Vlak, J., (1988) Bioreactor design for growth of shear-sensitive mammalian and insect cells. In: A. Mizrahi, editor. Advances in biotechnological processes. New York: Alan R. Liss.

Trinh, K., Gracia-Briones, M., Hink, F., Chalmers, J. J., (1994) Quantification of Damage to suspended Insect Cells as a Results of Bubble Rupture. *Biotechnology and Bioengineering* **43**: 37-45.

Tsubouchi, M., Yamasaki, N., Yanagisawa, K. (1985) Two-Phase Titration of Poly(oxyethylene) Nonionic Surfactants with Tetrakis(4-fluorophenyl)borate *Analytical Chemistry* **57**: 783-784.

Uchida, K., Emoto, K. Daleke, D. L., Inoue, K., Umeda, M. (1998) Induction of apoptosis by Phosphatidylserine. *Journal of Biochemistry* **123**: 1073-1078.

Van der Pol, L., Tramper, J. (1998) Shear sensitivity of animal cells from a culture-medium perspective. *TIBTECH* **16**: 323-328.

Wassall, S. R., Regina, C. McCabe, Y. Ehringer, W. D. et al. (1992) Effects of Dietary Fish Oil on Plasma High Density Lipoprotein. *The Journal of Biological Chemistry* **267**: 8168-8174.

Watt, F. (1991) Cell culture as a model for Cell differentiation. *FASEB* **5**: 287-294.

Weeratunge, N. (1993) Toxicological studies with Pluronic F-68 in In vitro Animal cell Culture. M.Sc. Thesis. University of Surrey. Guildford, Surrey, U.K.

Wichbold, R. (1972) The Determination of Nonionic Surfactants in Rivers and Waste Water. *Tenside Detergents* **9**: 173-177.

Wu, J. (1996) Insights into protective effects of medium additives on animal cells under fluid stresses: the hydrophobic interactions. *Cytotechnology*; **22**: 103-109.

Wu, J., Ruan, Q., Peter Lam, H. Y., (1997) Effects of Surface-active medium additives on insect cell surface hydrophobicity relating to cell protection against bubble damage. *Enzyme and Microbial Technology* **21**: 341-348.

Yamane, I., Kan, M., (1982) A Novel substitute (alpha-cyclodextrin) included with oleic acid and linoleic acids) for bovine serum-albumin in serum-free culture of mammalian cells. *In Vitro Cellular and Developmental Biology* **18**: 306-307

Yamaoka, T., Kondo, M., Honda, S., Iwahana, H. et al. (1997) Amidophophoribosyl-transferase Limits the Rate of Cell Growth-Linked de Novo Purine Biosynthesis in the Presence of Constant Capacity of Salvage Purine Biosynthesis. *The Journal of Biological Chemistry* **272**: 17719-17725.

Yang, M. (2001) Ammonia effects on CHO cell growth, metabolism, erthropoietin production and glycosylation. Ph.D. Thesis. University of Manitoba, Winnipeg, Manitoba, Canada.

Yuh-Fun, M., and Chung, H. C, (1997) Protein denaturation by combined effect of shear and air-liquid interface. *Biotechnology and Bioengineering*. **54**: 503-512.

Zaleska, M., Nagy, K., Floyd, R. (1989) Iron-Induced Lipid Peroxidation and Inhibition of Dopamine Synthesis in Striatum Synaptosomes. *Neurochemical Research* **14**: 597-605.

Zhang, J., Kalogerakis, N., Behie, L. (1994) Optimization of the phsiochemical parameters for the culture of Bombyx mori insect cells used in recombinant protein production. *Journal of Biotechnology* **33**: 249-258.

Zhang, S., Handa-Corrigan, A., Spier, R. E., (1992)[a] Oxygen Transfer Properties of Bubbles in Animal Cell Culture Media. *Biotechnology and Bioengineering* **40**: 252-259.

Zhang, Z., Al-Rubeai, M. Thomas, C. R. (1992)[b] Effect of Pluronic F-68 on the mechanical properties of mammalian cells. *Enzyme and Microbial Technology* **14**: 980-983.

Zhang, Z., Al-Rubeai, M., Thomas, C. R. (1992)[c]; Mechanical Properties of Hybridoma cells in Batch culture. *Biotechnology Letters* **14**: 11-16.

Appendix

Preliminary experiments for:

1) Choosing the optimal testing condition for viscometer experiments

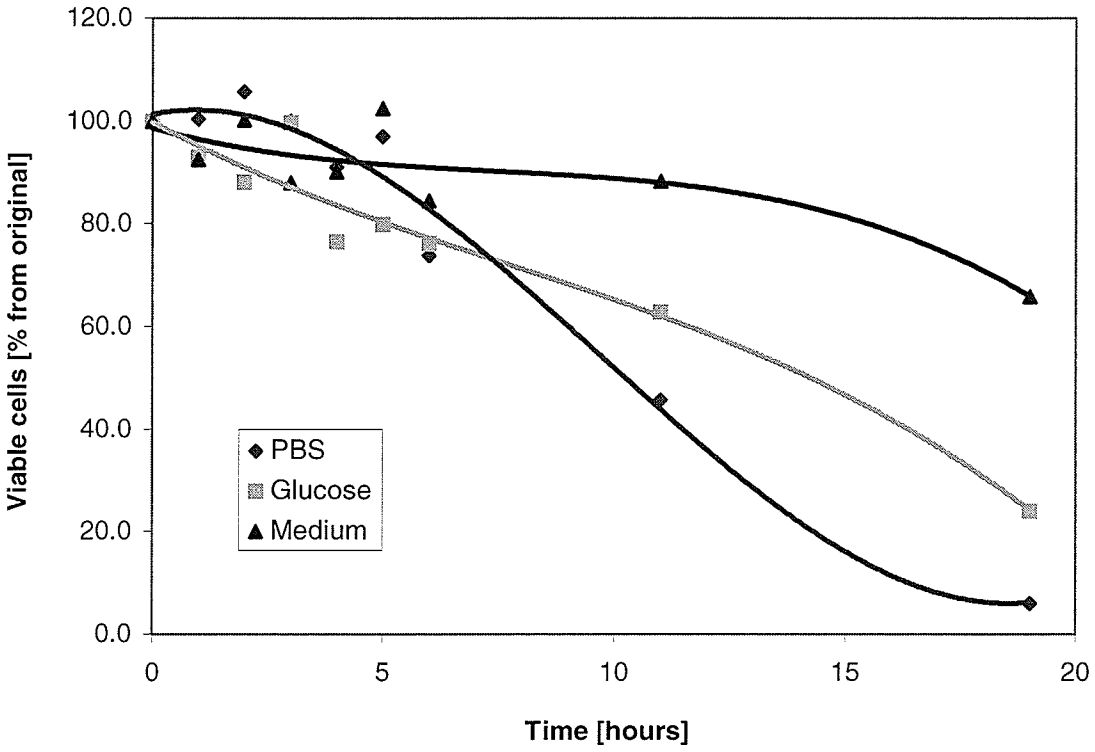


Figure A.1 Decay curve for CHO 81 cells (10^6 cell/ml) left standing at room temperature suspended in a 15 ml tube in PBS, PBS with 25 mM glucose or CNJsfm2.1 medium at room temperature (n=1).

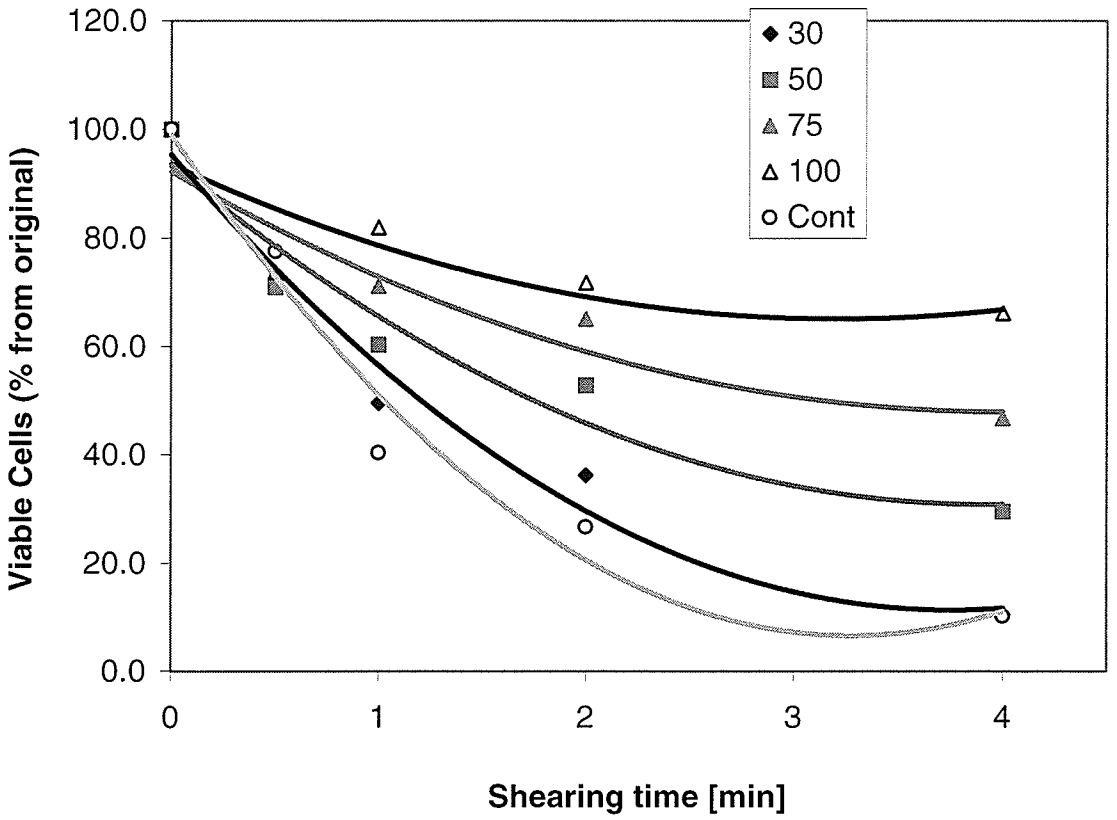
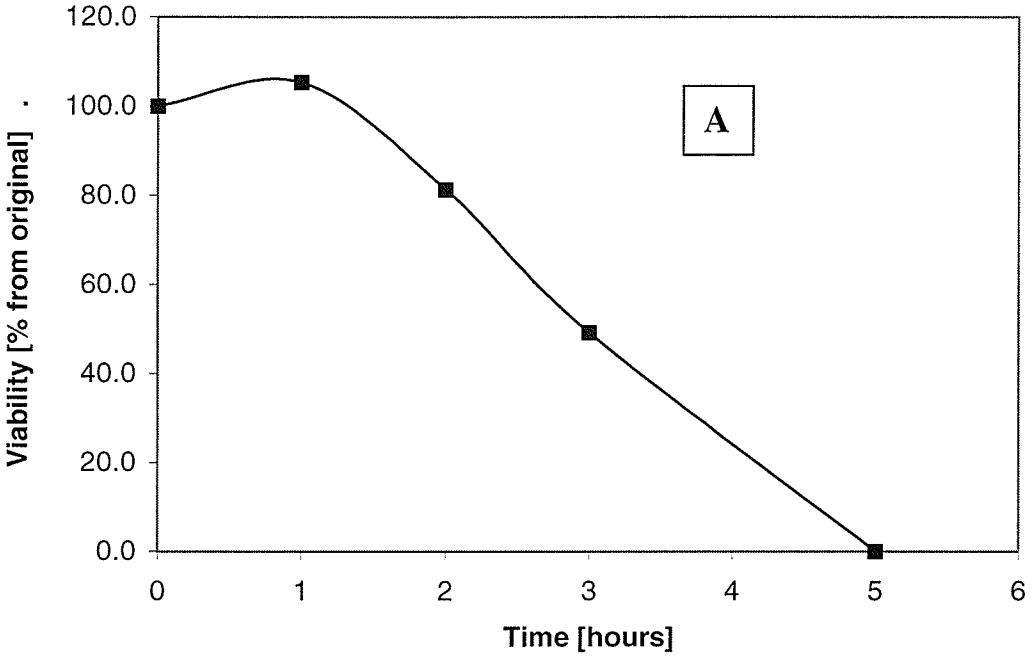


Figure A.2 Effect of trypsinization on the sensitivity of CHO to shear rates of 26,666 s^{-1} using Brookfield CAP 2000 viscometer. Cells were sheared in a culture medium immediately after 30, 50, 75, or 100 seconds of trypsinization. Control represents untrypsinized cells (n=1).

Trypsin appears to decrease the sensitivity of the cells to shear.

II) Choosing a suitable viscometer.



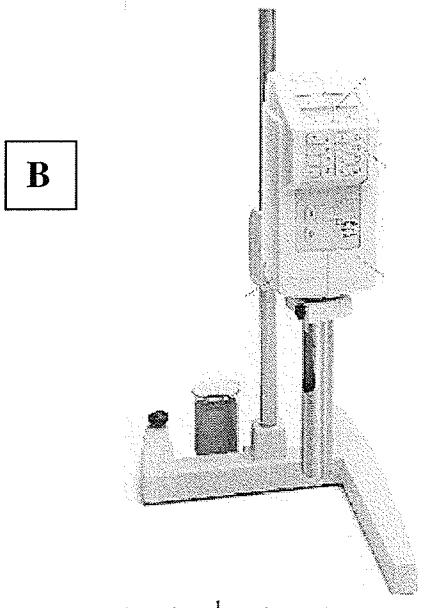
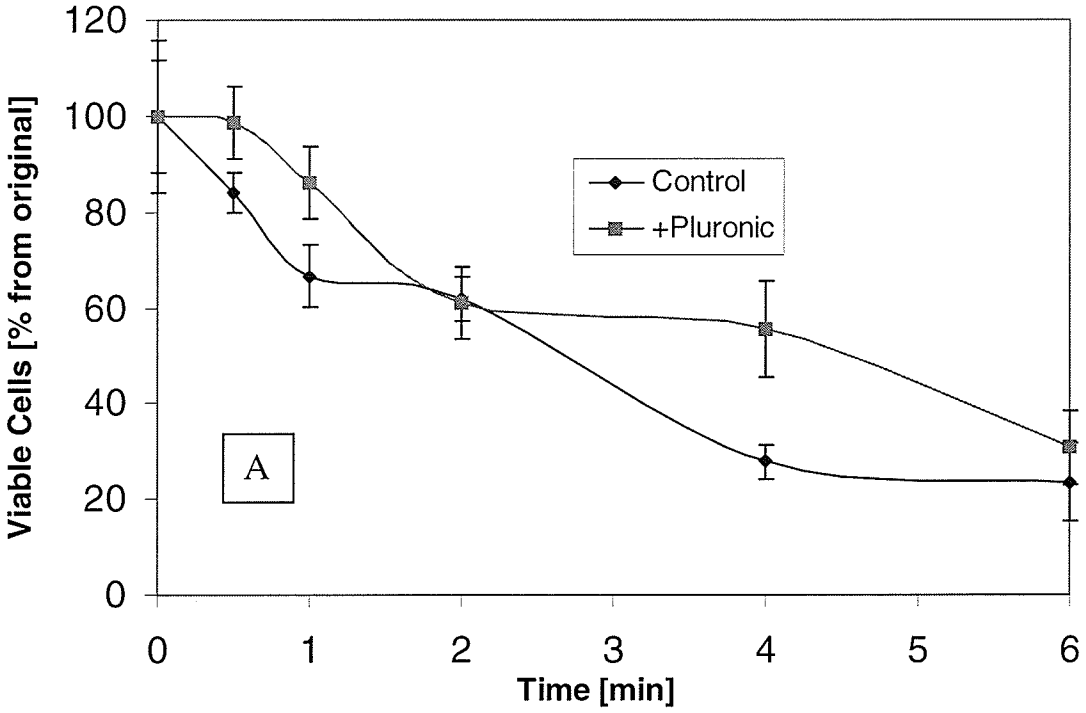


Figure A.3 A) Decay curve for CHO cells sheared at 3219 s^{-1} using the Rheomat 180. Cells were suspended in PBS at room temperature (n=1). B) Image of Rheomat 180 viscometer



B

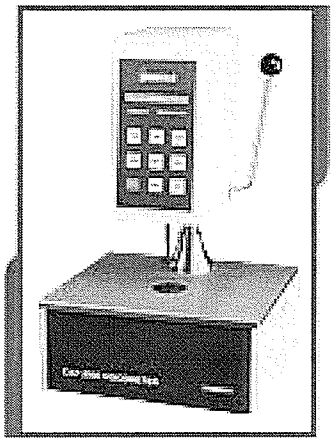
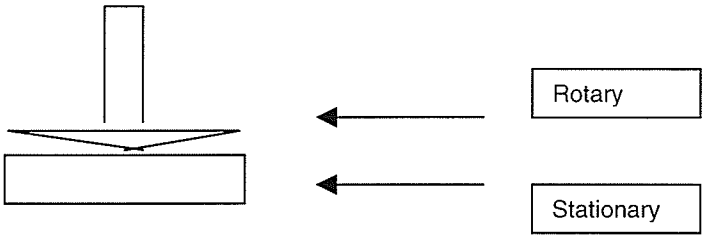


Figure A.4 A) Effect of Pluronic F68 on the sensitivity of CHO to shear rates of 26,666 s^{-1} using Brookfield CAP 2000 viscometer. Error bars represent the SEM (n=4). Cells were sheared in culture medium. B) Image of Brookfield CAP 2000 viscometer

A



B

Figure A.5 Rheolab MC1 Viscometer (from Paar Physica). A) The cone and plate of the viscometer B) An image of the instrument.

Rheolab MC1 viscometer was chosen over the Rheomat 180 and CAP 2000 viscometers for the experiments in this thesis. The reasons are discussed in chapter 5.

III) Washing unbound linoleic acid with PBS or BSA in PBS.

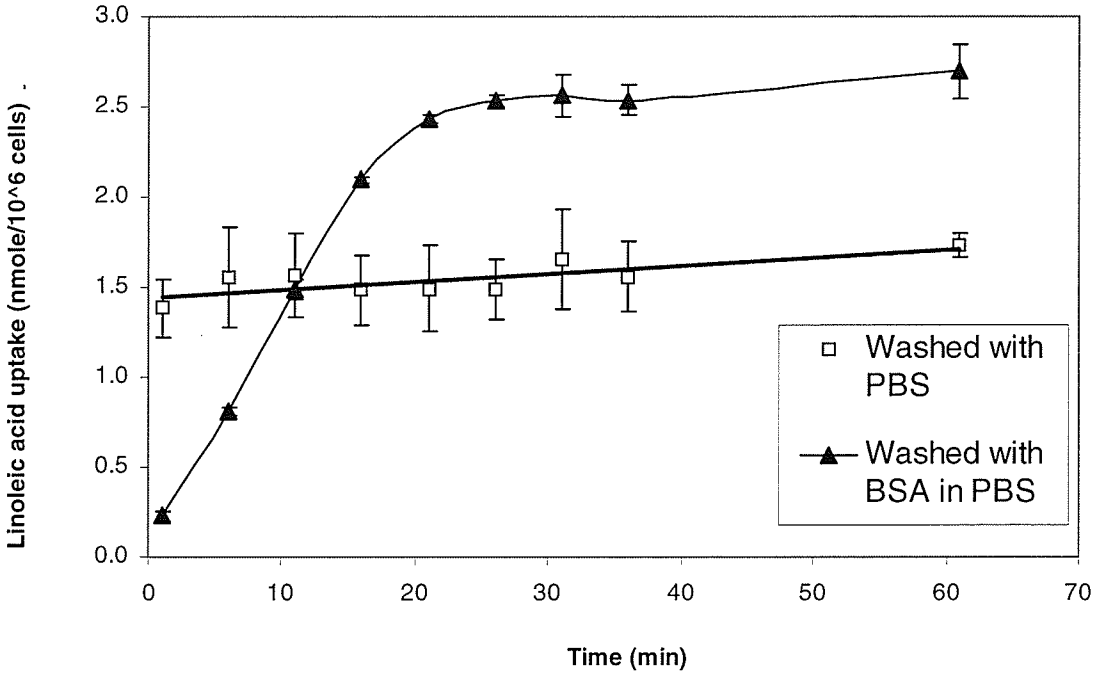


Figure A.6 Free linoleic acid uptake by hybridoma cells. Radioactivity was recorded with cells washed with PBS only or BSA (0.1 %) in PBS. Radioactivity was converted to equivalent linoleic acid taken up in nmoles (n=4).

ID:304 BULLER 14C 26 SEP 2000 20:04
USER: 5 COMMENT:LINOLEIC ACID
PRESET TIME : 2.00
DATA CALC : SL DPM H# :YES SAMPLE REPEATS: 1 PRINTER : STD
COUNT BLANK : NO IC# : NO REPLICATES : 1 RS232 : OFF
TWO PHASE : NO AQC : NO CYCLE REPEATS : 1
SCINTILLATOR: LIQUID LUMEX: NO LOW SAMPLE REJ: 0
LOW LEVEL : NO HALF LIFE CORRECTION DATE: none

ISOTOPE 1: 14C %ERROR: 0.00 FACTOR: 1.000000 BKG. SUB: 0

BACKGROUND QUENCH CURVE: Off COLOR QUENCH CORRECTION: On

Quench Limits Low: 3.619 High:327.17

SAM NO	POS	TIME MIN	H#	14C CPM	14C %ERROR	14C DPM	14C EFF-1	LUMEX %	ELAPSED TIME
1	1-1	2.00	86.5	29443.00	0.82	31369.09	93.86	0.00	2.49
2	1-2	2.00	83.7	29051.00	0.83	30918.70	93.96	0.00	5.05
3	1-3	2.00	84.1	28308.50	0.84	30133.09	93.94	0.00	7.62
4	1-4	2.00	84.5	28552.00	0.84	30396.84	93.93	0.00	10.18
5	1-5	2.00	87.5	29907.00	0.82	31875.92	93.82	0.00	12.77
6	1-6	2.00	87.7	31490.50	0.80	33565.86	93.82	0.00	15.35
7	1-7	2.00	90.0	30663.00	0.81	32713.33	93.73	0.00	17.92
8	1-8	2.00	84.2	29383.50	0.83	31278.29	93.94	0.00	20.49
9	1-9	2.00	85.7	28523.00	0.84	30380.10	93.89	0.00	23.06
10	1-10	2.00	86.1	28312.50	0.84	30160.19	93.87	0.00	25.62
11	1-11	2.00	87.2	32951.50	0.78	35116.33	93.84	0.00	28.21
12	1-12	2.00	88.9	30308.50	0.81	32320.68	93.77	0.00	30.79
13	2-1	2.00	88.8	31313.00	0.80	33390.50	93.78	0.00	33.46
14	2-2	2.00	87.8	30329.00	0.81	32329.54	93.81	0.00	36.05
15	2-3	2.00	87.3	31628.00	0.80	33708.02	93.83	0.00	38.64
16	2-4	2.00	85.9	32802.50	0.78	34940.80	93.88	0.00	41.22
17	2-5	2.00	112.4	20488.00	0.99	22069.42	92.83	0.00	43.78

University of Manitoba, Buller Building

Prof. Michael Butler laboratory team.