The Effect of Pluronic and Microcarriers on the Growth, Recombinant Protein Production and Glycosylation of CHO Cells

by

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Abstract

Various culture conditions enable the maximization of recombinant protein production from culture processes. Monitoring the productivity (volumetric and specific) gives us an idea of culture processes that are efficient for production of the protein products. However, there are many variables that need to be monitored in order to achieve proper operational platforms for production and for the assurance that the product formed is indeed similar or enhanced compared to usual culture processes used for the production.

A procedure was developed to increase recombinant protein production, particularly that of Interferon-beta (IFN-beta) and tissue plasminogen activator (tPA), that utilized the introduction of microcarriers. Volumetric protein yields were found to increase by up to 2.5 fold and specific productivity by up to 5.5 fold compared to corresponding suspension cultures of the respective proteins.

An important variable that was shown to be effective for decreasing aggregation of IFN-beta is the use of low temperature for the culture processes. The use of low temperature (32°C) in combination with microcarriers increased the volumetric product yield 5 fold compared to a conventional suspension 37°C batch bioreactor culture. The recombinant protein obtained from cultures where higher product yields were reached was tested for consistency in product glycosylation compared to that obtained in the suspension cultures.

The use of Pluronic F68 (PF68) in the culture medium in static cultures discourages the attachment of cells onto T-flask surfaces; this is shown to be a concentration dependent

effect where increased PF68 present decreases surface attachment. PF68 in stirred cultures promotes the increase in cell protection from shear stresses and proteolytic damage. Furthermore, the use of PF68 in culture processes does not affect the glycosylation compared to cultures not supplemented with it.

Abbreviations

ATCC	American Type Culture Collections
BHK	Baby Hamster Kidney
IFN-beta	Beta Interferon
BSA	Bovine Serum Albumin
CV	Coefficient of Variance
DHFR-MTX	Dihydrofolate reductase – methotrexate
ELISA	Enzyme linked Immuno Sorbent Assay
HPLC	High Performance Liquid Chromatograhy
HEK	Human Embryonic Kidney
IgG	Immunoglobulin G
kDa	Kilodalton
MAb	monoclonal Antibody
mRNA	messenger ribonucleic acid
NP-HPLC	Normal Phase HPLC
PBS	phosphate buffered saline
PF68	Pluronic F68
Qp	specific productivity
RP-HPLC	Reversed Phase HPLC
SFM	Serum Free Media
μ	specific growth rate
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
tPA	tissue plasminogen activator

WAX	Weak Anion Exchange

Amino Acids:

Asn	Asparagine
Ser	Serine
Thr	Threonine
Pro	Proline

Monosaccharides:

Fuc	Fucose
Gal	Galactose
Glc	Glucose
GlcNAc	N-acetylglucosamine
Man	Mannose
NeuAc	Sialic Acid (N-acetylneuraminic acid)

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Tharmalingam, T., K. Sunley, et al. (2008). "High yields of monomeric recombinant beta-interferon from macroporous microcarrier cultures under hypothermic conditions." Biotechnology Progress **24**(4): 832-838.

Chapter 1
1. Introduction

1.1. Mammalian cell culture

Transfected mammalian cell lines have been used to synthesize a wide range of compounds that can be extracted and purified for use as biopharmaceuticals. From 1986 to 2006 there has been an increase in the number of products that have been approved for production by mammalian cell processes and in the demand for these biopharmaceuticals (Butler, 2007). Due to the increasing demand, the maximization of productivity of these proteins is important to decrease costs of production. There have been many recombinant proteins that have been licensed for use as biologicals to treat human disorders, some of which include tissue plasminogen activator (tPA) and interferon-beta (IFN-beta).

This introduction will cover general topics encountered in mammalian cell culture, and then more specifically into subject areas covered in this thesis. The projects covered in this thesis are based on two parts - the use of microcarriers and pluronic F68 (PF68) in cell culture. The use of microcarriers in cell culture was to enhance recombinant protein production from CHO cells. The use of temperature shifts was to further enhance protein production and the effect of increased protein production was tested on the glycosylation of the protein. The use of PF68 is widely used as a culture additive in cell culture, and here we describe the uses and the effects of this component in cell media.

1.1.1. Cell line

The predominant cell line used for human recombinant protein production has been the Chinese Hamster Ovarian (CHO) cell line. CHO cells are well characterized and

deemed to be a stably transfected cell line (Derouazi *et al.*, 2006; Barnes and Dickson, 2006).

The CHO cell line was isolated from an inbred female adult Chinese hamster in 1958 (Puck *et al.*, 1958). Now the cell line is used in combination with dihydrofolate reductase (DHFR) as a selectable and amplifiable marker. Methotrexate (MTX) can be used to amplify genes such that high levels of recombinant proteins can be isolated. Fann *et al.* (2000) used methotrexate to amplify tPA produced by CHO cells. However, there are reports that suggest that amplified DHFR-CHO cell lines expressing recombinant proteins show long term culture instabilities in the absence of MTX (Kim, 1998; Browne and Al-Rubeai, 2007).

CHO cells have been used for recombinant protein production due to their ability to achieve glycosylation in a way similar to human cells. The cell line has been licensed and approved for the synthesis of therapeutic products. Many studies have been done on CHO cells that have been transfected to produce recombinant proteins such as erythropoietin (Restelli *et al.*, 2006), human growth hormone (hGH) (Keane *et al.*, 2003), interferon-beta (Rodriguez *et al.*, 2005; Spearman *et al.*, 2005), and tissue plasminogen activator (Andersen *et al.*, 2000; deZengotita *et al.*, 2002; Fann *et al.*, 2000; Senger and Karim, 2003).

1.1.2. Media Formulations

In an attempt to increase productivity and decrease costs, the change from serum based to serum free media (SFM) formulations has been made. Another reason for the

change from serum containing media to SFM is to eliminate adventitious agents such as viruses and other transmissible agents. As well, serum is chemically undefined and thus is subject to variation between batches. This variation results in the inconsistency of cell growth. Finally, serum is quite expensive and can compromise the extraction and purification procedures for cell secreted proteins.

For the production of bioactive recombinant proteins, the shift from serum free media to protein free media is being investigated (Chun *et al.*, 2007). SFM can contain hormones and growth factors to mimic the conditions of serum containing media. As well, SFM often contains insulin, transferrin, ethanolamine, and selenite (Butler, 2005). Protein free media will ensure the supply of a large quantity of bioactive proteins at a lower cost and further enhance the consistency of production of recombinant proteins. Furthermore, the movement towards protein free media facilitates the downstream purification of these recombinant proteins. Protein free media can also be optimized to each cell line and the physiological conditions in order to maximize growth and increase productivity (Murakami, 1990). Unlike serum containing media which may be utilized for a broad range of cell types and culture conditions, protein free media are generally highly-specific (Jayme, 1991).

The adaptation of cells to SFM (including protein free media) changes cell growth characteristics. Cells such as Chinese Hamster Ovary (CHO) cells and Baby Hamster Kidney (BHK) cells naturally grow adherent to surfaces; however, with the introduction of these cells into SFM, the cells are found to grow in suspension.

1.2. Cell Culture

When trying to maximize the productivity of an animal cell line from an industrial standpoint, the cultures are often scaled up from 1 litre to 1000 litres in stirred tank bioreactors. These bioreactors stir the media and cells, evenly distributing nutrients, making sure that no gradients form and that cell concentrations are uniform throughout the tank. The rotating impellors generate shear forces or shear stresses in the bioreactor, which has the potential to kill cells (Dusting *et al.*, 2006; Wang *et al.*, 2005).

1.2.1. Oxygen Supply

Another source of stress on the cells is oxygen sparging into the culture, which is required to introduce sufficient levels of oxygen within a bioreactor culture. The oxygen supply within the bioreactor culture depreciates as the culture grows, and is influenced by the growth rate of the cells. The specific oxygen consumption rate of mammalian cells varies from 0.05 to 0.5 pmol/cell/h for a culture (Eyer *et al.*, 1995; Jorjani and Ozturk, 1999).

In an attempt to control oxygen levels in a bioreactor, Kilburn *et al.* (2000), sparged oxygen into a culture of LS mouse cells. The absence of sparging throughout the culture led to a maximum number of cells; sparging from the beginning of the culture and then stopping at day 3, created an increased lag phase, but cells did proliferate after some time to levels comparable (but not as high) to those of the nonsparged culture. The sparged culture, after having grown for 2 days, showed a sharp decline in cell numbers. This experiment shows the effect of physical damage in bioreactor cultures. This result

was duplicated using CRL 8018 hybridoma cells in an agitated bioreactor by Kunas *et al.* (1990).

The specific oxygen uptake rate is influenced by the culture variables at a given time. Jorjani and Ozturk (1999) showed that the specific oxygen uptake rate is not influenced by the cell density when cell concentrations are constant between 1 and 20 million cells/mL. However, they do show that specific cell lines have different specific oxygen consumption rates and when compared to murine hybridoma cell lines, CHO cells had the highest consumption. Under a lower temperature regime, BHK cells and hybridoma cells were found to have a 10 to 20 % decreased specific oxygen consumption rate (Jorjani and Ozturk, 1999; Kyung *et al.*, 1994; Ruffieux *et al.*, 1998).

The sparging of oxygen into culture medium creates air bubbles to which cells attach. The bubbles eventually reach the liquid-air interface at which the interfacial tension of the medium is high and the bubbles burst. The attached cells become entrapped within the liquid surrounding the bubble and eventually die within the "death zone" due to high shear forces (Jordan *et al.*, 1994; Trinh *et al.*, 1994).

Trinh *et al.* (1994) attempted to quantify the number of cells killed and the locations of the cells (*Spodoptera frugiperda* (SF-9)) relative to the bubbles, and found that a significant number of these insect cells were killed as a result of the rupture of a single bubble. Using a method where bubbles were released at the base of a column, then watched as they rise, Dey *et al.* (1997) also reported the attachment of hybridoma cells to bubbles with no surfactant present. This visualization allowed them to see that as bubbles rise, they create a surface to which cells can attach. The cells die when the bubbles burst.

By checking the viability at the end of the column it was reported that the number of dead cells increased compared to the same treatment in the presence of the surfactant.

1.2.2. Shear Forces

Shear stress (τ) is defined by the ratio of force per area that the force acts on. By knowing the viscosity of the fluid (β) and the shear rate (γ) formed by the velocity gradient between the two surfaces, we can determine the shear forces that are applied on cells. Shear force is the force that is exerted on cells.

$$\tau = \beta \gamma$$

The use of water in the medium results in the viscosity to be 1 cPoise. Since 1 cPoise is equal to 1 dyne s cm^{-2} and 1 dyne s cm^{-2} is equal to 0.1 Pascale s (Pa s), substituting the known values into the equations shear stress can be calculated.

Shear stress is believed to be a function of viscosity, surface tension, length of the smallest turbulent eddy, regions of converging flow, and the generation of a deep vortex associated with bubble entrainment (Van der Pol and Tramper, 1998). The effects of shear stress on a cell can be predicted by the type of cell and species, cell wall thickness, size and morphology of cell, intensity of shear stress, growth history, growth environment, growth medium, growth rate, growth state, type and concentration of shear protective agents (Chisti, 1999).

Several examples of shear stress experiments can be found in the literature. For example, Al-Rubeai *et al.* (1995) found that increasing the agitation rate of hybridoma

spinner cultures from 100 rpm to 600 rpm decreased cell viability, and a progressive loss was found from 84 % on day 1 to 63 % on day 2 to 56 % on day 3 at 600 rpm. A study that looked at baby hamster kidney cells (BHK21) found that increasing shear stress to greater than 15 Nm^{-2} for more than 1 hour decreased the viability (Ludwig *et al.*, 1992). Smith *et al.* (1987) found that mouse hybridoma B45 cells had a decrease from $2.9x10^5$ to $0.9x10^5$ cells/mL when exposed to a shear rate of $1240s^{-1}$ for 14 hours. Damaged cells were covered with blebs and filamentous microvilli while the control cells were clear of any cellular components (Smith *et al.*, 1987).

Shear stress can affect all types of cells, although animal cells are weaker (due to the absence of a cell wall), they are not the only type of cell to be affected by shear stresses. Sun *et al.* (1999) examined plant cells, *Taxus cuspidata*, in a rotating wall vessel bioreactor with exposure to different shear stresses. The cells grew well when exposed to lower shear stresses of 0 to $5.6x10^{-3}$ Nm^{-2}, but when exposed to higher shear stresses of $8.9x10^{-3}$ and $11.1x10^{-3}$ Nm^{-2} the cells had a decreased lag phase and increased growth rate with a decreased time span of exponential growth.

Shear stress can be detrimental to cells in the absence of protectants such as PF68. Shear forces can be created by the rotating of the impellor, the cells bumping into one another in a stirred culture, and the bubbles being released in the culture during sparging. Numerous studies have looked at methods to reduce shear forces on mammalian cells.

1.3. Media Additives

Mammalian cell media often contain components that would work to protect cells from high shear forces. In the past, with addition of serum, there were conditioning factors that worked to protect cells from high shear situations, and thus decreased cell damage. In SFM, the addition of detergents and other chemicals is common to protect cells from high shear damage. A well known component – PF68, is commonly added to media to protect cells from this damage; however, there are other components that have been used such as Methocel E50, Methocel E4M, polyvinylalcohol (PVA) and methylcellulose.

1.4. Pluronic F68

PF68 is a nonionic surfactant which has been extensively studied as a protective agent added into SFM. PF68 consists of a polymerized structure of ethylene oxide and propylene oxide chains (Ahmed *et al.*, 2001).

$$HO-(CH_2CH_2O)_a-(CH_1\,CH_2O)_b-(CH_2\,CH_2O)_c-H$$
$$\qquad\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\qquad CH_3$$

The average molecular weight of PF68 is 8400 Da, of which ethylene oxide makes up 80 %. Pluronic molecules are categorized by numbers, depending on the values of a, b, and c. PF68 has average values for a, b, and c of 75, 30, and 75, respectively. There are different varieties of pluronic that are expressed by the letter and numbers that follow pluronic. The central poly(oxypropylene) block of the molecule is lipophilic and the poly(oxyethylene) blocks are hydrophilic.

1.4.1. The function of Pluronic

The functions of Pluronics, Methocel E50, Methocel E4M, polyvinylalcohol (PVA) and methylcellulose are believed to be similar, but they have been used in experiments to compare their ability to protect. The mode of action of PF68 is unclear, as there are two major theories that are used to explain its function:

Mechanical Effect – where PF68 works to reduce cell to bubble attachment, thus inhibiting the cells from coming in close contact to shear forces due to bubble rupture.

Biological Effect –where PF68 is able to somehow repair damage that the cells are exposed to by plugging any damaged areas of the cell membrane (Marquis *et al.*, 1989).

1.4.1.1. Mechanical Functions

The presence of PF68 in small quantities was found to decrease the velocity of rising bubbles and CHO cell aggregates or single hybridoma cells did not adsorb to their surfaces. Jordan *et al.* (1994) suggested that the degree of saturation determines whether or not cells adhere to the surface of bubbles. Other studies indicate that PF68 prevents the attachment of cells to bubbles thereby protecting damage caused by aeration into a bioreactor (Jordan *et al.*, 1994; Meier *et al.*, 1999; Michaels *et al.*, 1995). PF68 was also shown to decrease the rupture time of the bubble to less than 10 seconds, thus decreasing the shear force being produced by the bubble rupture and the interfacial tension of the medium (Chattopadhyay *et al.*, 1995; Trinh *et al.* 1994).

Michaels *et al.* (1995) found that the protective mechanisms of PF68 and other media additives, such as polyvinylalcohol and methocel, reduced cell to bubble

attachment and that the fluid mechanical forces near the rupturing bubble are reduced and cell resistance to shear is increased. The protective effect of PF68 was also reported with plant and insect cell lines (Handa-Corrigan *et al.*, 1989). Sowana *et al.* (2002) showed a significant improvement in the biological responses for wild carrot cell cultures grown in a medium supplemented with PF68, where cell breakage was reduced considerably.

1.4.1.2. Biological Functions

Palomares *et al.* (2000) suggests that the effects of PF68 on cell resistance to shear even after removal from the culture medium indicates the existence of a stable and direct interaction between PF68 and Sf9 insect cells. A plant cell line (*Nicotiana tabacum*) that was exposed to PF68 had increased productivity and it was suggested that PF68 enhanced the permeability of the cell wall thus increasing the recombinant protein being released (Lee and Kim, 2002). Gigout *et al.* (2008) viewed with fluorescently labeled PF68 that the molecules are taken up by the endocytic pathway and are transported to the lysosomes. These observations imply that the protective effects of PF68 are due to the uptake of the surfactant by cells.

1.4.2. Pluronic and its effects on protein production

The addition of pluronics (of various kinds) has been found to inhibit adhesion of cells to the surfaces of hydrophobic regions. Biran *et al.* (1999) found that Pluronic F108 (PF108) was useful for the inhibition of adhesion and growth of cortical astrocytes on a model adhesive surface. Looking at extracellular matrix proteins and the ability to support neuronal attachment and outgrowth, they found that there was no difference in the specific production of extracellular matrix proteins produced between those cells

exposed to PF108 and those that had no exposure. This indicates that the extracellular matrix proteins being synthesized by the cell are only important in the first few days of the culture (Biran *et al.*, 1999). Another study looked at the deterrence of cell adhesion of hepatocytes and fibroblasts by use of PF108. The authors suggested that cell adhesion is dependent on cell type and culture conditions. PF127 has use in the pharmaceutical industry and has been found to decrease liposome to cell adhesion using CHO cells (Chandaroy *et al.*, 2002).

The presence of PF68 for the duration of a culture period has been shown to increase the productivity of the protein recombinantly produced by the cell line. Keane *et al.* (2003) reported that CHO cells that were exposed to shear stress of a 0.10 Nm^{-2} in the absence of PF68 ceased production of human growth hormone. When the cells that were exposed to the shear force were then exposed to 0.2 % PF68, the production of human growth hormone continued at rates comparable to the initial one. Another study that looked at a recombinant protein, murine granulocyte macrophage - colony stimulating factor (mGM-CSF), produced by plant cells, *Nicotiana tabacum*, reported an increase in the production of the protein produced by the cell line in the presence of PF68 (Lee *et al.*, 2002). It is presumed that PF68 enhanced the permeability of the cell wall, thus allowing the mGM-CSF to be released. The presence of PF68, in a concentration dependent manner in *Morinda citrifolia* cultures was found to manipulate the cell membrane such that a secondary metabolite, anthraquinone, would be released (Bassetti *et al.*, 1995). However, the effect of PF68 on cell productivity seems to be cell line dependent as PF68 had no effect on the production of the recombinant protein, erythropoietin, produced by CHO cells (Ghebeh *et al.*, 2002).

1.4.3. Non-ionic surfactants and protein stabilization

A low concentration of non-ionic surfactants has been shown to stabilize proteins, due to the prevention or reduction of protein surface adsorption and reduction of aggregation due to a low critical micellar concentration (CMC). The presence of non-ionic surfactants such as Tween 20, Tween 80, and PF68 reduce chemical degradation of proteins. PF68 has been shown to inhibit thermally-induced aggregation and precipitation of recombinant human growth hormone (Katakam *et al.*, 1997).

1.5. Microcarriers

CHO cells naturally grow adherent to the surface of T-flasks, when grown in serum containing medium. However, with the introduction of PF68 into serum free formulations, these cells grow in suspension. To allow the cells to grow in their natural state, there has been the development of microporous and macroporous microcarriers. The trade names include Cytodex (microporous) and Cytopore, Cytoline, and Cultispher G (macroporous). Macroporous microcarriers - Cytopore, Cytoline, and Cultispher G are composed of mesh like balls in which cells become entrapped and grow. However, Cytoline is often used in a fluidized bed bioreactor (Cytopilot) as it is a weighted bead. Microporous microcarriers, Cytodex, in some cases collagen-coated, are beads that cells can adhere to. Microcarrier cultures allow the cells to grow in pseudosuspension cultures within bioreactors and spinners without allowing the cells to be exposed to shear forces caused by the impellors turning or sparging (Cherry *et al.*, 1988; Koynov *et al.*, 2007). Furthermore, shear rates were found to decline with the increasing load of microcarriers, but were found to not be affected by the microcarrier diameter or density (Grima *et al.*, 1997).

Cell adhesion to microcarriers is attained by the adsorption of attachment factors to the culture surface, contact between the cells and the surface, attachment of the cells to the surface, and spreading of the attached cells (GE Heathcare, 2005). The attachment of cells depends on the divalent cations (Ca^{2+}, Mn^{2+}) present and proteins in the medium or adsorbed onto the surface of cells (Grinnell, 1978). The attachment of cells is induced by fibronectin (Fn), which is present in serum. However, with the movement to serum free based media, there is a requirement for the addition of fibronectin to induce cell attachment onto surfaces (Hughes *et al.*, 1979; Virtanen *et al.*, 1982).

There are various intracellular attachment proteins involved in connecting the cytoskeleton with transmembrane linker proteins, which in turn bind to transmembrane proteins of other cells, creating the cell-to-cell or cell-to-substratum adherence. Cell-to-cell contact and adherence is achieved by adhesion molecules, like cadherins, in particular α and β catenins (Tsuiji *et al.*, 2007). Cell-to-substratum contact is mediated by adhesion molecules like integrins and interacting molecules like talin and vinculin (Yamada and Miyamoto, 1995). Other proteins form focal adhesions, and are found to recruit a large number of proteins to this region, in particular talin, vinculin, and paxillin, when cell-to-cell adhesion responses are targeted. The recruitment of these proteins often leads to morphological changes which contribute to speading, migration, or cell signaling, often indicating that cell attachment is occurring.

Cell-to-cell attachment was visualized by image correlation microscopy in a CHO cell model system, where integrin microclustering was detected in adherent cells (Ginsberg *et al.*, 2005). Integrins are heterodimeric transmembrane molecules that consist

of α and β subunits. The subunits can occur in active and inactive conformations, where in an inactive conformation the integrins have a low affinity for ligands, and thus disallow cell attachment. In an active conformation, ligand binding occurs and thus activation of signal cascades leading to the assembly of multiprotein complexes at the site of cell adhesion occurs. Cells with the deletion of the highly conserved β1 integrin gene led to defects in adhesion, proliferation, survival and polarity.

Recent studies have shown the importance of an IPP complex in cell adherence; Integrin-linked kinase (ILK), PINCH (particularly interesting Cys-His-rich protein), and Parvin proteins exist in a complex that would occur with cell-to-substratum attachment (Legate *et al.*, 2006). In the complex, ILK would bind to PINCH and parvin. However, it is poorly understood as to how PINCH regulates cell to cell adhesion. Studies on the adhesion by PINCH show that this protein may regulate adhesion in an indirect manner.

1.5.1. Cytodex

The different variants of Cytodex include Cytodex 1, 2 and 3. Cytodex 1 microcarrier is based on a cross-linked dextran matrix with positively-charged N,N diethylaminoethyl (DEAE) groups, which are found throughout the entire matrix of the microcarrier. Cytodex 2 is similar to Cytodex 1; however has a larger charge variation. Cytodex 3 consists of a surface layer of denatured collagen covalently bound to a matrix of cross-linked dextran. Collagen coated surfaces are used to grow cells that are normally difficult to grow in culture as the cells are more adherent to the collagen-coated surface, which in turn, promotes their growth.

1.5.2. Cytopore

There are different variations of Cytopore - Cytopore 1 and Cytopore 2. The two different types of Cytopore have the same structure in that they both are macroporous, and allow cells to enter the large pores and take refuge within.

The macroporous matrix supplies nutrients to the cells from both apical and basolateral sides relative to the cell. Once cells are found within the microcarriers they are able to grow in layers, and produce recombinant proteins, without being exposed to the shear forces that they would be exposed to when growing in suspension. The primary difference between Cytopore 1 and Cytopore 2 is that Cytopore 1 has a charge density of 1.1 meq/g while Cytopore 2 has a charge density of 1.8 meq/g.

Macroporous microcarriers were developed to allow cells to grow to high cell densities within the pores and protect them from fluid damage caused by the media flowing throughout the bioreactor or spinner culture. Xiao *et al.* (1999) reported that a higher stirring speed can be used (from 80 to 100 rpm) when using Cytopore - as less damage would be experienced at these higher speeds. In contrast, with Cytodex cultures, the higher rotational speeds would cause more damage, as more shear forces are experienced the cells on the surface of the bead.

There have also been other macroporous microcarriers reported for mammalian cell culture. These include Cytoline, Cultisphere G, and Collagen gel particles. The use of Cytoline in culture was reported to increase the production of erythropoietin from CHO cells by 2-fold compared to a suspension culture (Wang *et al.* 2002). Furthermore, the

production of an osteoblast derived antiviral protein from CHO cells was enhanced by 5.5-fold compared to a suspension culture (Kong *et al.* 1999). Yamaguchi *et al.* (1997) showed a 30 to 60-fold increase in volumetric monoclonal antibody production from BHK cells in an immobilized cell culture in collagen gel articles compared to a suspension culture.

The protection of the cells within the mesh of the Cytopore allows the cells to produce a stable recombinant protein with proper glycosylation and folding. Watson *et al.* (1994a) reported a difference in glycosylation in CHO cells producing recombinant human tissue kallikrein. The production of kallikrein within Cytopore cultures was enhanced, due to the increase in cellular growth. However, there appeared to be a decrease in sialylation in the Cytopore culture and more complex oligosaccharides in suspension cultures. Another study on the glycosylation of erythropoietin (EPO) recombinantly produced by CHO cells in a Cytoline culture found no difference in glycosylation when compared to a suspension culture (Wang *et al.*, 2002). Cytopore cultures of CHO cells producing recombinant IFN-beta showed no difference in glycosylation when comparing Cytopore 1 cultures to suspension cultures (Spearman *et al.*, 2005).

Vero cells that were grown in the presence of macroporous microcarriers were able to grow to high cell densities (Lim *et al.*, 1992; Yokomizo *et al.*, 2004). The porous microcarriers supported much higher cell growth compared to solid microcarriers due to the protection of cells from shear forces in the mesh of the microcarrier. The microcarrier in these studies is composed of cross-linked cellulose substituted with positively charged

N,N-diethylaminoethyl (DEAE) groups. For comparison, the surface area provided by Cytopore is 2.8 m^2/g while that provided by Cytodex is 0.6 m^2/g (Xiao *et al.*, 1999).

For anchorage-dependent cells, the greater surface area offered within the macroporous microcarriers can allow a significantly higher cell concentration in a culture and the potential for higher viral yields. In some cases higher viral yields through the use of macroporous microcarriers have been reported (Nikolai and Hu, 1992). However, in other cases the high cell/ bead loading may limit accessibility of the virus to the cells (Berry *et al.*, 1999). These variable observations suggest the productivity of viruses from such culture systems may depend upon the specific strategy of virus infection and progression in culture (Yokomizo *et al.*, 2004).

1.6. Recombinant Proteins

There are many factors that have to be monitored when producing recombinant proteins from mammalian cells. Although scaling up production capacity would increase protein yields, there must be the assurance that the product will be consistent with high stability. Protein stability is important to ensure that pharmaceuticals made from the isolated protein are stable and not subject to degradation prior to administration to a patient.

There are many factors that determine protein stability. Some of these factors are temperature, pH, presence of salts and metal ions, shaking or shearing, and the concentration of the protein (Wang *et al.*, 2002). A higher temperature can lead to protein instability and can accelerate chemical degradation. Proteins native to thermophilic

organisms are found to have a high molecular stability due to greater hydrophobic interactions, greater molecular packing, more hydrogen bonding, more salt bridging, loss of surface loops, more helix forming amino acids, and restricted N-terminus mobility (Wang *et al.*, 2002).

Protein aggregation is a serious problem that affects recombinant proteins. For the production of therapeutics, the presence of aggregates of any type is typically considered to be undesirable because of the concern that the aggregates may lead to an immunogenic reaction or may cause adverse events on administration (Cromwell *et al.*, 2006). The aggregates of protein can be soluble, ie. not visible as discrete particles and not removed by a 0.22 μm filter. Insoluble aggregates may be removed by filtration and are often visible to the unaided eye. However, both soluble and insoluble aggregates may be problematic for the production and use of recombinant proteins (Cromwell *et al.*, 2006).

Protein aggregation causes intermolecular association of partially denatured protein chains. Aggregation is induced by temperature, ionic strength, vortexing, surface and interface adsorption. Protein aggregation occurs commonly when proteins are overexpressed in foreign organisms, i.e. bacterial systems as *E. coli*, with the high concentration of material leading to the formation of inclusion bodies (Yon, 2001).

One important issue with protein aggregation is glycosylation. Glycosylation is important for protein stability, as it can affect protein activity, antigenicity, solubility, and proteolytic resistance. A properly glycosylated protein has increased stability as it is able

to form hydrogen bonds with a polypeptide backbone or surface hydrophilic amino acids and steric interactions with adjacent peptide residues.

1.6.1. Interferon-beta

Type 1 (alpha and beta) interferons are used to treat several diseases, including chronic viral hepatitis, lymphoproliferative disorders, and cancer. IFN-beta is classified as type II, which refers to its action through a common receptor. All type II interferons have five helices labeled A to E. The helices are left handed with a disulfide bridge extended from cysteine 31 of loop AB to cysteine 141 of loop DE (refer to Figure 1.1 for structure).

Human IFN-beta is an important cytokine which is secreted by fibroblasts in response to a viral infection or exposure to double stranded RNA (Honda *et al.*, 2003; Kawai and Akira, 2006). The secreted protein is normally 166 amino acids long with a glycosylation site at position 80. Human IFN-beta is glycosylated with a biantennary complex-type oligosaccharide with fucosylated and nonfucosylated trimannosyl core and Siaα2 →3 Galβ1 → 4 GlcNAcβ1 (Kagawa *et al.*, 1988). However, depending on the origin of the recombinant protein the interferon can be glycosylated (IFN-beta1a) or non-glycosylated (IFN-beta1b) (Giovannoni *et al.*, 2002; Conradt *et al.*, 1987).

IFN-beta produced by CHO cells is glycosylated at position 80 with a similar carbohydrate moiety, as that found in humans, but with the absence of a 2, 4 branched triantennary oligosaccharide, an increase in the quantity of 2,6 branched triantennary oligosaccharides and the partial involvement of N-acetyl lactosamine repeating unit

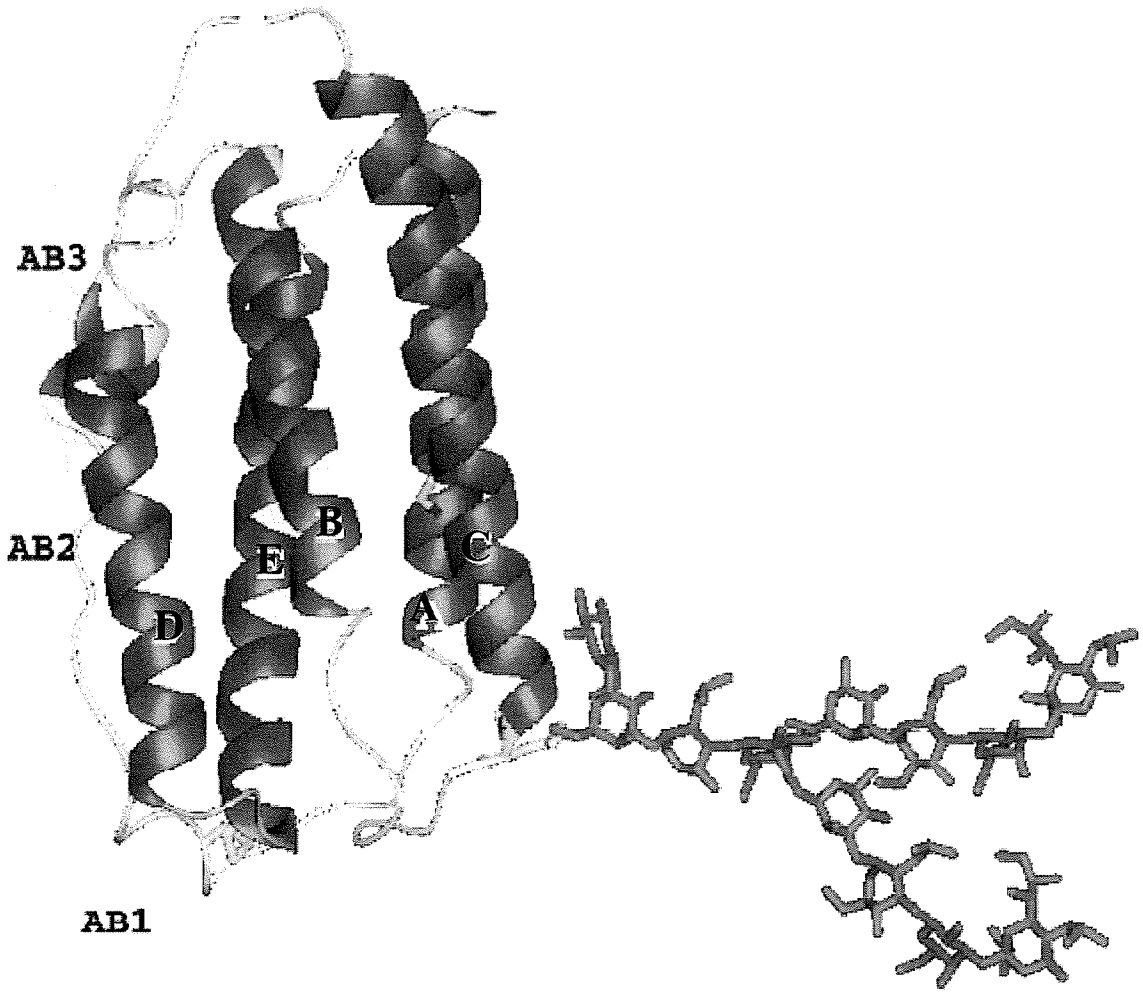

Figure 1.1: The structure of HuIFN-beta. Ribbon representation of the polypeptide chain, with full-size carbohydrate shown. Helices are labelled from A to E. Taken with permission from Karpusas (1998).

(Kagawa *et al.*, 1988). The major glycan chain in recombinant IFN-beta is a biantennary complex type, containing an α1-6 linked fucose on the peptide proximal GlcNAc residue and two α2-3 linked NeuAc on terminal galactose residues (Spearman *et al.*, 2005). *E. coli* produced recombinant IFN-beta is the nonglycosylated form, with methionine 1 deleted and cysteine 17 mutated to serine.

The lack of glycosylation has been shown to decrease the solubility (Conradt *et al.*, 1987), and induce the aggregation of IFN-beta (Runkel *et al.*, 1998). Furthermore the loss of sialic acid reduced the activity of IFN-beta (Utsumi *et al.*, 1995). Dissing-Olesen *et al.* (2008) reported that the removal of sialic acid from the IFN-beta prior to treatment reduced the efficiency of the protein, thus implying that the sialylation is important.

The IFN-beta produced by murine sources has a molecular weight of 25 kDa whereas *E. coli* produces a product of 17.5 kDa IFN-beta due to the lack of glycosylation. The two products are commercially available as Avonex® with CHO cell origins and Betaseron® with *E. coli* source. The Avonex® recombinant IFN-beta-1a has about 10-fold higher specific activity due to the presence of the glycosylated site which also allows it to be less immunogenic. IFN-beta-1 b (Betaseron) was approved for the treatment of relapsing and remitting multiple sclerosis in 1993 (Reingold, 1996).

1.6.2. Tissue plasminogen activator

Tissue plasminogen activator (tPA) is a thrombolytic agent. tPA is involved in a coagulation and fibrinolysis cascade that becomes activated when tPA cleaves serine protease to form zymogen plasminogen. The active chain of tPA has a molecular weight

of 70 kDa. tPA consists of 527 amino acids and 17 disulfide bonds. The tPA is arranged into 5 domains: finger (which is homologous to type 1 repeats of fibronectin), growth factor, kringle 1, kringle 2, and catalytic domain (Refer to Figure 1.2 for structure). Each kringle contains three loops held together by disulfide bridges. The catalysis domain contains the serine protease triad His 322, Asp 371 and Ser 478 (Stephens, 1993).

The cleavage of tPA, at the Arg275 and Ile276 peptide bond, by the action of the plasmin product allows tPA to be a more active, two-chained protease held together by a disulfide bond. Two chain tPA has similar enzymatic activity but is more susceptible to protease inhibitors *in vivo* (Wittwer and Howard 1990). tPA is naturally found in human blood at a concentration of 5 to 10 ng/mL and has a half life of 5 to 10 minutes.

There are two variable types of tPA (tPA I and tPA II) differing in the possible sites of glycosylation. There are a maximum of 4 glycosylation sites – 3 possible N-glycosylated sites and 1 O-glycosylated site. The 3 possible N-glycosylated sites are Asn 184, Asn 448, and Asn 17. All tPA produced within mammalian cells (recombinant tPA) contain the N-glycosylated chain of high mannose oligosaccharides at Asn 117 and a complex glycan at position Asn 448 (Pfeiffer *et al.*, 1994). Lubiniecki *et al.* (1994) found that Asn184 can have a complex multiantennary glycan form or can be unoccupied. When Asn184 is not glycosylated it forms the less active type II tPA and when it is glycosylated it forms the active type I tPA. The O-linked fucose residue is attached to threonine (Thr) 61.

Figure 1.2: The structure of tPA, showing the 2 kringle fragments, and 17 disulfide bonds. Disulfide linkages are indicated with black bars between cysteines. N-linked glycosylation sites are represented as black Y-shaped structures at Asn 117, Asn 184, and Asn 448. The arrow indicates the plasmin cleavage site (Arg 275) for conversion of single-chain TPA to two-chain TPA. Modified from Benedict *et al.* (2005).

Type I t-PA has been observed to display reduced clot lysis activity and reduced fibrin binding compared with type II t-PA (Einarsson *et al.*, 1985; Wittwer *et al.*, 1989). However, this is offset with an increased half life as type I single chained tPA is more resistant to plasmin catalysed conversion to two chained tPA than type II single chained tPA. It appears that the glycosylation site at Asn 184 inhibits the conversion reaction (Wittwer and Howard, 1990). It has been noted that the type of tPA (type I or type II) production is linked to the growth phase of the cell (G_o/G_i) for fed batch CHO cells producing recombinant tPA (Senger and Karim, 2003). As well, Senger and Karim (2003) found that high shear stresses triggered the CHO cells to produce less volumetric tPA. Of the tPA produced, type II tPA was more present within the culture media than type I tPA.

tPA can be recombinantly produced in *E. coli*, fungi such as *Aspergillus niger (A. niger)* (Weibe *et al.*, 2001), and mammalian cells such as CHO cells (Kimura and Miller, 1997; Lin *et al.*, 1993), Bowes melanoma cell line, mouse epithelial (C127) cells and human placental cells.

The glycosylation status of the tPA protein determines the clearance from circulation, thus heavily glycosylated tPA is more likely to be circulated and have a decreased half-life. The protein produced in *E. coli* is just as functional and equivalent in activity when compared to tPA produced in mammalian cell lines with glycosylated sites. However, the use of *E. coli* for recombinant protein production is not proficient, as the inactive protein is enclosed in inclusion bodies from which it has to isolated. Once it has been isolated from the cell, the protein has to be reactivated by *in vitro* adaptation of known folding

reactions (Mattes, 2001). The production of tPA within *E. coli* is low, as the organism has been known to produce 100 mg tPA/L/hour of the nonsecreted protein (inactive) and 10 to 100 μg tPA/L/hour of the active form (Qiu *et al.*, 1998).

tPA production in *A. niger* results in the production of low quantities of tPA. The organism produced low quantities of nonfungal proteins, as there is incorrect folding or processing of protein. This causes an upregulation of unfolded protein response and therefore proteolytic degradation. As well, fungi and *Saccharomyces cerevisiae* tend to hyperglycosylate recombinant proteins. *A. niger* has been known to produce 12 to 25 mg tPA/L (Wiebe *et al.*, 2001).

As the pharmaceutical is prepared and ready for use, the stability of tPA would have a major impact on the use of the protein. Wiernikowski *et al.* (2000) looked at the long term storage of tPA protein derived from CHO cells. The recombinant protein was found to be stable for up to 6 months at -80°C. However, the study looked at the stability at -30°C and found that samples of tPA (into 2 mg / 2 mL) were stable for only 22 weeks at this temperature.

Growth conditions have an impact on the stability of tPA. Altamirano *et al.* (2000) reported that tPA isolated from a two day culture with a viability of 92 % had a half life of 43 days, whereas the half life of tPA from media collected from a six day culture (viability of 71 %) was 15 days. Altamirano *et al.* (2000) also found that growing CHO cells producing recombinant tPA in the presence of tPA decreased production rates, indicating that there could be saturation of the secretory pathway. Comparing four clones

of CHO cells producing tPA, Fann *et al.* (2000) found that two high producers and two lower producers had a decrease of 60 % in the production of tPA after 139 days. Furthermore, the presence of methotrexate gave two cell lines the stability to produce tPA at a constant rate and two cell lines showed a slightly lower production (35 % and 40 % reduction).

There is a challenge to maximize productivity of recombinant proteins produced by mammalian cells. Through modifications of growth conditions, i.e. through media additions or growth on microcarriers, cells can be manipulated to increase productivity.

1.7. Glycosylation

There are three types of oligosaccharides that can be added to proteins to make up a glycoprotein: N-linked glycan, O-linked glycan and glycosyl phosphatidylinositol (GPI) anchors. Briefly, N-linked glycans are attached to a glycoprotein at a sequon, which consists of Asparagine – X – Serine / Threonine (Asn-X-Ser/Thr), where X can be any amino acid residue except Proline (Pro). Although glycans are added to the Asn residue in a sequon, not all sequons may be occupied. O-linked glycans, are classified as mucins. They involve a glycan attached via an O-glycosidic bond to a Ser or Thr residue. A GPI anchor is a preformed glycolipid structure that is transferred to the C-terminal region of a protein.

1.7.1. N-linked Glycans

Mammalian cells utilize the glycosylation pathway to add glycans to proteins – resulting in glycoproteins. The pathway begins by the synthesis of a polyisoprenoid lipid-

linked precursor oligosaccharide ($Glucose_3$ $Mannose_9$ N-Acetylglucosamine$_2$ - $Glc_3Man_9GlcNAc_2$) from the carrier lipid dolichol phosphate to the asparagine of a sequon of the protein (as shown in Figure 1.3).

First the protein, which is translated by the ribosome is transferred to the endoplasmic reticulum (ER) where it begins to be glycosylated and disulphide bridges are formed. There are chaperones and folding enzymes within the ER to assist in the folding of the glycoprotein. The protein must be folded correctly prior to exiting the ER.

The transfer of the oligosaccharide ($GlcNAc_2Man_9Glc_3$) from the Dolichol-P to the glycoprotein is mediated by the oligosaccharyl transferase (OST). Trimming of the oligosaccharide to $Man_8GlcNAc_2$ must occur within the ER, prior to displacement to the golgi, and these reactions are mediated by glucosidase I and II (αGI and αGII) and ER mannosidase (αM). The movement of the glycoprotein from the ER to the cis-, median-, and trans-golgi occurs via transport vesicles.

Once the glycoprotein reaches the Cis-Golgi, 3 mannose residues are cleaved off by the enzyme α-mannosidase I (αMI), resulting in $Man_5GlcNAc_2$. The glycoprotein is then transported to the median-golgi where further processing occurs. There is the addition of a GlcNAc by N-acteylglucosaminyl transferase I (GnTI), the removal of two more mannose residues by αMannosidase II (αMII), and then further additions of GlcNAc and Fucose (Fuc) (by GnTII and fucosyl transferase; FucT). The oligosaccharide is further processed in the trans-golgi by additions of galactose (by galactosyl transferase, GalT) and sialic acids (by sialyl transferase, ST). Incomplete processing may lead to varying

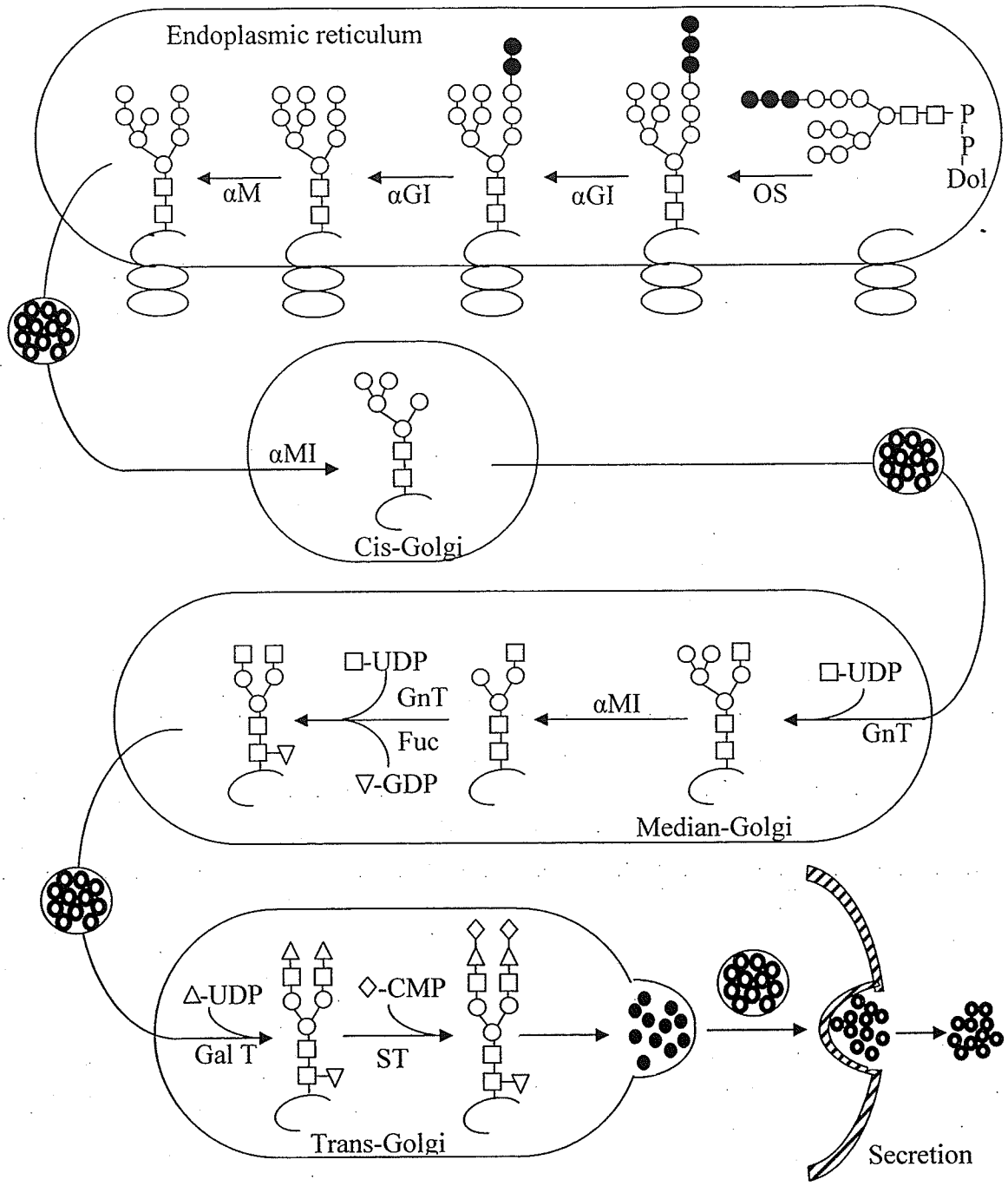

Figure 1.3. The pathway for the biosynthesis of N-linked glycans. Biosynthesis starts in the rough endoplasmic reticulum and proceeds through the *cis*-, median-, and *trans*-Golgi apparatus, before the glycoprotein is secreted. Some of the enzymes involved are: oligosaccharyl transferase (OST); α-glucosidase I & II (αG I & II); ER-mannosidase (αM); α-mannosidase I & II (αM I & II); *N*-acetylglucosaminyl transferase I & II (GnT I & II); fucosyl transferase (FucT); galactosyl transferase (GalT); sialyl transferase (ST). □-Gn; ○-Man; ●-Glc; ▽-Fuc; △-Gal; ◊-SA. Modified from Kornfeld and Kornfeld (1985).

types of N-glycans on proteins, whereby high mannose structures or hybrid glycans can be produced.

N-linked glycans can be classified as high mannose, hybrid, and complex-type. These three classifications involve a common core structure composed of $Man_3GlcNAc_2Asn$ and different outer branches. A high mannose oligosaccharide would incorporate the core structure with 2 to 6 additional mannoses while a complex incorporates the core with 2 or more outer branches containing N-acetyl glucosamine (GlcNAc), galactose (Gal), and sialic acids (NeuAc). A hybrid is a combination of both these structures (Refer to Wildt and Gerngross, 2005).

Mammalian cells (non-human) are the optimal choice for recombinant protein production; however, the glycosylation is also found to differ compared to human glycosylation (Figure 1.4 E & F). Most mammals (humans) express the enzyme α1-3-galactosyltransferase which generates Galα1,3-Galβ1,4-GlcNAc residues; however mouse cell lines are incapable of forming the α1,3- linkage and thus may induce mild human immune responses (Stephens *et al.* 1995)

Strategies have been employed to engineer the host CHO cell line with extra glycosyltransferase enzymes (e.g. α2,6-sialyltransferase) normally inactive in CHO cells but present in humans (Bragonzi *et al.*, 2000). As well there has been the transfection of the β1, 4- N-acetyl-glucosaminyltransferase III to reduce IgG antibody-dependent cellular cytotoxicity (ADCC) (Ferrara *et al.*, 2006). Feeding key carbohydrate precursors such as N-acetylmannosamine (ManNAc) to improve sialylation has also been proposed

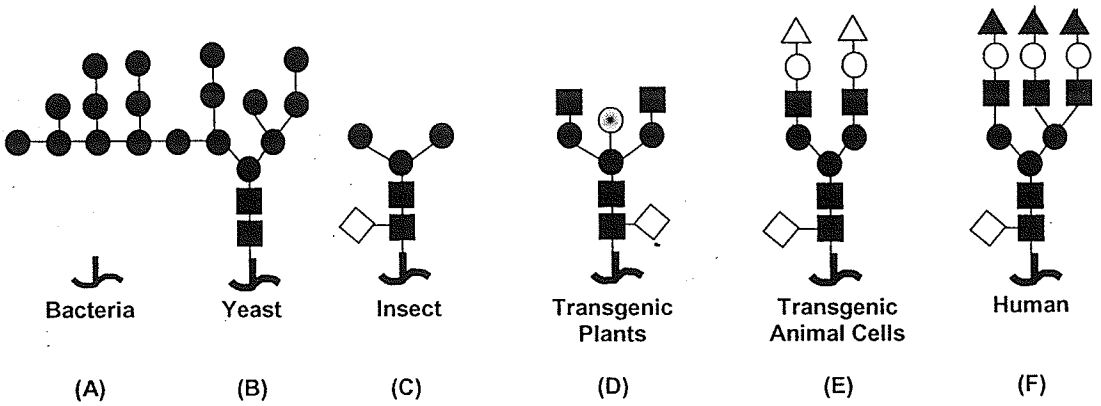

Figure 1.4: The differences in glycosylation by different host cells. Where ⅄ represents the peptide, ■= N-acetylglucosamine (Glc-NAc), ●= Mannose, ○= Galactose, ◇= Fucose, △= N-glycolylneuraminic acid, ▲=N-acetylneuraminic acid, and ◉= Xylose. Modified from Jenkins *et al.*, (1996).

and tested (Gu and Wang 1998; Hamilton *et al.*, 2006; Campbell *et al.*, 2007). Gu and Wang (1998) fed up to 20 mM of ManNAc, a sialic acid precursor, to CHO cells producing interferon-γ and observed an increase in percentage sialylation from 60 to 80%. However, increasing the ManNAc concentration up to 40 mM showed no further improvement in the sialylation.

Other cell types used for large scale production of recombinant proteins may produce varied glycosylation. Bacteria used for recombinant protein synthesis are incapable of glycosylating protein (Figure 1.4A). Yeast N-glycosylation is of the high mannose type(Figure 1.4B), which causes a short half life *in vivo* and thus decreases the efficacy of recombinant human therapeutic proteins produced in yeast (Jenkins *et al.*, 1996). However, in the past few years, there is the report of the development of yeast strains capable of performing hybrid N-glycosylation (Choi *et al.*, 2003), and the production of

complex oligosaccharides (Hamilton *et al.*, 2003). Furthermore, recently there has been a report on *Pichia pastoris* cell lines capable of secreting terminally sialylated, complex, bi-antennary glycoproteins (Hamilton *et al.*, 2006).

Plant cells are capable of glycosylation; however, their proteins tend to lack sialic acid residues (Figure 1.4D). Also plants tend to have core α1-3 linked fucose instead of an α1-6 linked fucose (Gomord *et al.*, 2005). As well, the bisecting β1-4 N-acetylglucosamine is generally substituted by a β1-2 xylose (Ma and Hein, 1995).

Insect cells that produce recombinant proteins have the inability to synthesize glycoproteins with authentic glycosylation (Figure 1.4C). The glycans lack complex N-linked oligosaccharides containing galactose and terminal sialic acids due to the absence of galactosyltransferase and sialyltransferase. As well, there are insufficient levels of terminal glycosyltransferases to convert most N-linked side chains to complex forms. The membrane bound N-acetylglucosaminidase processes $GlcNAcMan_3GlcNAc_2$ to $Man_3GlcNAc_2$, and thus the highly processed N-linked oligosaccharide on insect cell glycoproteins consist of $Man_3GlcNAc_2$ (+/- Fuc) (Hollister, 1998).

The glycosylation found in transgenic mammals (Figure 1.4 E) is similar to that found on human proteins (Figure 1.4 F), with the exception of the sialic acids. N-glycolylneuraminic acid (NeuGc) is a derivative of the sialic acid N-acetylneuraminic acid (NeuAc) and is rarely found on human glycoproteins. Low levels of NeuGc are tolerated in recombinant proteins (1%) but higher levels may cause and immunogenic

response (Noguchi *et al.*, 1995). NeuAc is the predominant sialic acid found on CHO cell glycoproteins; however, CHO cells are capable of producing NeuGc (Hokke *et al.*, 1995).

The N-linked oligosaccharides play multiple roles in the folding of proteins and are required to stabilize proteins and make proteins more soluble thus resulting in less aggregation. The lack of glycosylation results in aggregated forms of the protein, thus resulting in the lack of usable protein.

1.7.2. O-linked Glycans

The most common type of O-linked glycans contain an initial N-acetyl galactosamine (GalNAc) residue attached to a peptide chain via serine or threonine residues. The majority of O-linked glycans contain a core GalNAc sugar, and to these there are 8 possible second sugar linkages, thus giving 8 GalNAc core structures (Figure 1.5). O-Linked glycans tend to be very heterogeneous and thus they are classified by their core structure. However, the termini of O-linked glycans may include Gal, GlcNAc, GalNAc, Fuc, or sialic acid. The process of O-glycosylation occurs in the golgi. Mucins, or other O-linked glycans, include glucosamine, xylose, galactose, fucose, or mannose as the initial sugar bound to the ser/thr residues.

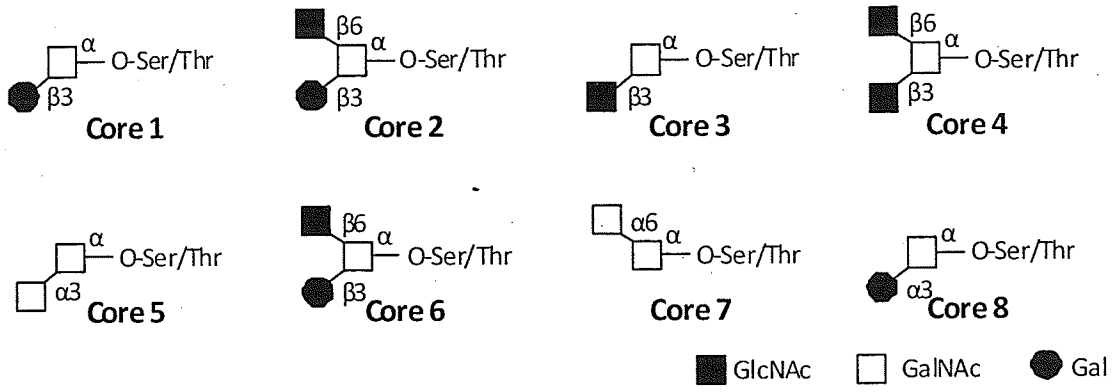

Figure 1.5: Core O-glycans: Structures of the eight known core types with Ser/Thr attached GalNAc sugars. Modified from Butler (2005).

1.7.3. The importance of glycosylation

Glycosylation can influence the activity, clearance rate, antigenicity, immunogenicity, stability and solubility of proteins (Brooks, 2004; Kimura and Miller, 1997). In studies looking at the physical stability of erthyropoeitin due to the glycans, the presence of a glycan on proteins of mammalian cell origin allowed the protein to be soluble at a low pH (2.5), whereas the *E. coli* expressed protein precipitated at pH 5 and below. As well, the protein, isolated from mammalian sources was soluble at a higher temperature (90°C), whereas that expressed by *E. coli* precipitated at about 40°C (Nahri *et al.*, 1991).

Glycan heterogeneity can be altered by many cultural environmental factors, such as nutrient starvation (Curling *et al.*, 1990; Hayter *et al.*, 1992; Kontoravdi *et al.*, 2007; LeFloch *et al.*, 2006), metabolic waste accumulation (Yang and Butler, 2000a; Yang and Butler, 2000b; Yang and Butler, 2002), culture viability (Andersen *et al.*, 2000), pH (Borys *et al.*, 1993; Curling *et al.*, 1990; Muething *et al.*, 2003; Wagner, 1986), oxygen

concentration (Serrato *et al.*, 2004), and temperature (Andersen *et al.*, 2000). Thus there is a requirement to monitor the impact of these culture variables in order to have consistent glycosylation, and consistent glycoprotein formation under varying culture conditions.

The choice of cell line also greatly influences the glycosylation pattern of the recombinant glycoprotein, since cells differ in their complement of glycosyltransferases enzymes that control the glycosylation process (Baker *et al.* 2001; Jenkins *et al.* 1996). The function of a particular glycan in a protein may be as complex and diverse as the function of a particular amino acid and be important for proper protein folding, solubility, recognition, ligand binding, protein stability, or it may have no function at all and be completely replaceable (Wyss and Wagner, 1996). The structure of glycans is influenced by the competition of glycosylation enzymes for the same substrate, substrate specificity of these enzymes and substrate availability (Khmelnitsky, 2004).

Sialic acid is a terminal sugar and a charged monosaccharide present on glycoprotein oligosaccharides. The presence or absence of sialic acid can affect many glycoprotein properties, including specific activity (Goldwasser *et al.*, 1974; Imai *et al.*, 1990); antigenicity (Hevey *et al.*, 1997; Schauer, 1988; Stephenson *et al.*, 2003); resistance to protease attack (Aquino, 1984; Goldwasser *et al.*, 1974), and resistance to thermal denaturation (Goldwasser *et al.*, 1974). Sialic acid containing carbohydrates are highly hydrophilic structures and increase solubility of proteins by shielding hydrophobic residues (Sinclair and Elliott, 2005). It was suggested that the hepatic asialoglycoprotein receptor (ASGPR) bound desialylated glycoproteins, targeting them for degradation.

Thus, the rate of desialylation of glycoproteins was hypothesized to control their clearance (Sinclair and Elliott, 2005). Thus, the presence of terminal sialic acid is a desirable feature for human therapeutic proteins requiring a long circulatory half-life.

1.7.4. Biopharmaceutical manufacturing

The paradigm of routine biomanufacturing is to ensure batch-to-batch consistency in product purity, efficacy and safety. Alterations to structural features, such as protein fold or post-translational modification (e.g. glycosylation or phosphorylation) can alter product efficacy. It is thus important to identify and monitor these crucial parameters during production to assess the effect of changes in cell culture conditions on product quality.

1.8. Thesis Objectives:

The objective of this thesis is to study the effects of Microcarriers and PF68 on the growth of CHO cells producing the recombinant proteins. IFN-beta and tPA were chosen as good representatives of recombinant proteins that are important biopharmaceuticals. PF68 and Microcarriers are important for protecting cells against high shear forces in bioreactor cultures. Thus it is important to analyze the different effects of these two culture variables on the growth of cells, and more importantly the productivity and product consistency.

1. Comparison of the growth and productivity of CHO cells producing recombinant IFN-beta and tPA on microporous and macroporous microcarriers

The growth and productivity of CHO cells in microporous and macroporous microcarrier cultures was investigated. Recombinant protein production rates differ with different culture conditions. There is the need to increase the productivity of cells such that the cost of production can be decreased. Increasing the cell density within a culture by use of microcarriers (Cytopore and Cytodex) led to higher levels of proteins within a culture.

2. The incorporation of Cytopore into a hypothermic culture to enhance productivity

A major trend in the production and growth of stable cell lines, involves the achievement of controlled proliferation. Controlled proliferation stops cells from progressing in the cell cycle from the transition from Gap 1 to the Synthesis phase. The cells are able to survive with minimum nutrient requirements thus keeping the cells in a production phase. Part of this thesis explores one way that controlled proliferation can be achieved by using low temperature. The use of low temperature in bioreactor cultures was investigated by following the growth of the IFN-beta producing cell line and the effect on the production of IFN-beta.

3. Analysis of the effects of Cytopore and temperature shift on the glycosylation of IFN-beta

Growing cells at high cell densities should increase the level of recombinant proteins. However, increasing the level of protein in a culture sometimes causes the cells to stop producing and degeneration of the protein occurs (Altamirano *et al.*, 2000; Fann *et al.*, 2000). Protein titers can be measured by ELISA; however ELISA titre measurement

offers little data on the protein product itself. The analysis of the glycosylation would give us information about the product and the consistency of different preparations and thus enable us to conclude on efficient culture parameters that should be used for the production of IFN-beta.

4. To study the effect of PF68 on CHO cell growth, recombinant protein production and glycosylation.

PF68 is a media additive used to help protect cells from high shear forces. The use of PF68 within stationary T-flask cultures was monitored, as well as the growth and productivity of CHO cells producing IFN-beta and tPA in larger scaled cultures. In this study we looked at the production of recombinant IFN-beta and tPA in the presence of different concentrations of PF68. Furthermore, the effects of PF68 on the glycosylation of IFN-beta were also explored.

5. To study the protective effect of PF68 in the presence of bubbles and in a bubble free environment

Shear forces are detrimental to animal cells, as the cells do not have walls, causing these cells to be weaker than plant or bacterial cells. Palomares *et al.* (2000) found that a sample of insect cells vortexed in the presence of PF68 had higher cell viability than cells vortexed in the absence of PF68. Using a very high shear rates we caused damage to CHO cells (two different cell lines) and tested the protection ability of PF68 at varying concentrations. The protective effect of PF68 was also tested on proteolytic damage by trypsinization.

Chapter 2
2. General Materials and Methods

2.1. Cell Culture

2.1.1. Cell lines

Chinese Hamster Ovary (CHO) cells were derived from a transformed non-tumorogenic hamster ovary cell in 1957. A subclone, CHO-K1, was transfected with the IFN-beta gene by Cangene Corporation, Winnipeg, Canada. Many clones were provided to Prof. M. Butler at the University of Manitoba, of which CHO 674 were used for the current studies.

The recombinant human tPA producing Chinese Hamster Ovary (Castro *et al.*) cell line was purchased from American Type Culture Collection (ATCC). CHO cell line (ATCC No. CRL-9606) was produced by transfecting CHO cells with a plasmid (pETPFR) to produce a system in which tPA can be produced.

Both CHO cell lines have the ability to grow in suspension in Biogro Serum Free Media (SFM) (Winnipeg, Canada). However they can be induced to grow in an anchorage dependent fashion with a fibroblast-like morphology.

2.1.2. Chemicals

All chemicals and reagents were obtained from the Sigma Chemical Company unless otherwise indicated. All additions to the culture medium were of cell culture grade or of the highest purity available.

2.1.3. Culture Medium

Cells were cultured in Biogro CHO Serum Free Medium (CHO-SFM). The medium contained low levels of protein (less than 20 mg/l) and PF68 (0.1 % w/v), unless otherwise mentioned.

2.2. Culturing Methods

2.2.1. Sub-culturing

CHO cells were subcultured in 75 cm^2 T-flasks for stationary cultures with a working volume of 12 mL. Cells were subcultured every 3 to 4 days with an inoculation density of $1x10^5$ cells/mL. Cells that were adherent required trypsinization for detachment of cells from the flasks prior to subculturing. The trypsinization protocol involved removal of cultured media from the flasks, washing of adherent cells with Phosphate Buffered Saline (PBS) (Gibco, 21600-10) with 1mM ethylenediaminetetraacetic acid (EDTA) and then incubation with trypsin in PBS (0.2 mg/mL) for 5 to 10 minutes at room temperature. The flasks were then tapped vigorously to dislodge cells. The trypsin reaction was stopped by addition of Soybean trypsin inhibitor (0.2 mg/mL). The cell suspension was then transferred to a 15 mL centrifuge tube and centrifuged for 5 minutes at 300 xg. The supernatant was removed and the cell pellet was resuspended in 10 mL of Biogro-CHO-SFM for counting in a haemocytometer, and then inoculated at $1x10^5$ cells/mL.

The cells were grown in an incubator with 5 % CO_2 with the caps loosened to allow for equilibration with the atmosphere and at 37°C. All cell culture steps outlined were done under aseptic conditions in a NuAire Laminar Flow Hood. All media, trypsin,

trypsin inhibitor, and other reagents used for cell culture were sterilized by 0.2 μm membrane filtration (Pall, Ann Arbor, MI).

2.2.2. Cell counting

2.2.2.1. trypan blue

Equal volumes of a cell sample and 0.2 % trypan blue solution (in PBS) were mixed for up to 3 minutes prior to sample enumeration in a Neubauer haemocytometer. This method enabled us to estimate the viable cells within the culture, as the live cells do not take up the dyes, and dead cells stain blue indicating the uptake of the trypan blue.

2.2.2.2. Crystal Violet

Equal volumes of a cell sample and 0.5 % crystal violet solution (0.2 M citric acid solution with Triton detergent (2 % v/v) with 0.5 % (w/v) crystal violet) were mixed. The cells were lysed in the crystal violet solution at 37°C for up to 3 hours. The nuclei were then enumerated using a Neubauer haemocytometer.

2.2.2.3. Haemocytometer

All reported counts are an average of 2 counts of the same sample. In each counting event, 4 boxes of the haemocytometer were counted and the cell concentration was calculated according to the formula as follows:

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

2.2.2.4. Specific growth rates

The specific growth rate (μ) measures the rate of cell number increase. The formula used for its calculation was:

$$\mu\ (h^{-1}) = \frac{\ln N - \ln No}{t}$$

Where No is the initial cell concentration, N is the cell concentration at time t, and t is the time (in hours) elapsed from the start of the cell growth.

2.2.3. Experimental Cultures

Cultures were set up in spinner flasks when experimental conditions required. The spinner flasks available were 100 mL, 250 mL, 500 mL and 1000 mL cultures. The cells were inoculated at a density of $1x10^5$ cells/mL (unless otherwise mentioned). The spinners were set to spin at a rotational speed of 40 rpm, except in shear exposure experiments (where rotational speeds were set to 100 rpm or higher, as indicated). The cells were grown in an incubator with 5 % CO_2 with the caps loosened to allow for equilibration with the atmosphere and at 37°C.

Cell culture in a bioreactor culture was established in a 3 L Applikon bioreactor with a working volume of 2 L of medium and an inoculum of $1.0x10^5$ cells/mL. Cultures were maintained at pH 7.1, dissolved oxygen at 50 % air saturation and a temperature of 37°C. The agitation speed was maintained at 100 rpm with a marine impeller. A daily sample was taken until viability declined below 60 %. A minimum of 200 mL of supernatant was removed from cultures for glycosylation analysis, where required.

2.3. Protein Detection

The specific rates of production (Qp) of β-IFN were calculated from plots of product concentration during the growth phase against the integral of values of the growth curve (IVC).

2.3.1. ELISA for the detection of IFN-beta

Polyclonal rabbit anti-human antibodies were purchased from Biogenesis (Poole, UK) and monoclonal antibodies were purchased from Chemicon (Temecula, USA). The recombinant human IFN-beta standard, goat anti-mouse IgG alkaline phosphatase conjugate, and detection substrate were purchased from Sigma Canada.

All supernatants were analyzed by ELISA with a method developed by our laboratory. The samples were harvested, then immediately stored at -70°C to prevent any aggregation after freezing. Polyclonal antibody (0.2 μg/mL) in 0.1M sodium bicarbonate buffer was used to coat the 96-well plates overnight. The plates were washed between each step 3 times with Tris Buffered Saline with Tween (TBS with 0.1 % Tween, TBS-T). The plates were then blocked for 1 hour with 3 % BSA/TBS-T at room temperature. Meanwhile samples were prepared by diluting culture supernatant samples 1:50 or 1:100 and denaturation of the samples to ensure complete detection was occurring. The denaturation was an adaptation of the denaturation of western blot samples where 1 μL of SDS and 1 μL of β-mercaptoethanol was added to 100 μL of supernatant and then boiled for 5 minutes at 95°C. The samples were added to the 96-well plates and serially diluted. Mouse monoclonal anti-human IFN-beta diluted at 1:1000 (1 μg/ml) was incubated for 1 hour at room temperature. Alkaline-phosphatase conjugated with anti-mouse IgG

(1:15,000 of 5 μg/mL) was diluted in 1 % BSA TBS-T then introduced to the washed plate for 1 hour at room temperature. The colour was developed by addition of p-nitrophenyl phosphate substrate and incubation overnight at 4°C. The absorbance was taken at 405nm by Softmax ELISA reader.

The concentration of the USB non-glycosylated IFN-beta standard was calibrated with a glycosylated recombinant Human Interferon beta 1-a from Oxford Biotechnologies (OBT 1546). This allowed for the conversion of IFN units/mL to IFN mg/L, where 100,000 units/mL corresponded to 0.2 IFN-beta mg/L.

2.3.2. ELISA for the detection of tPA

Monoclonal antibodies were purchased from Cedarlane Laboratories (Burlington, Canada) and Polyclonal rabbit anti-human antibodies and recombinant human tPA standard were purchased from Cortex Biochem (Concord, USA). The goat anti-mouse IgG alkaline phosphatase conjugate and detection p-nitrophenyl phosphate substrate were purchased from Sigma Canada.

All supernatants were analyzed by ELISA, as developed in our laboratory. The samples were harvested then immediately stored in -70°C to prevent any aggregation or degradation after freezing. Monoclonal antibody (0.91 μg/mL) in 0.1 M sodium bicarbonate buffer was used to coat the 96 well plates overnight at 4°C. The plates were washed between each step 3 times with TBS-T (TBS with 0.1 % Tween). The plates were then blocked for 1 hour with 3 % BSA/TBS-T at room temperature. Samples of cultures were prepared by diluting them 1:50 or 1:100. The samples were added to the 96 well

plates and serially diluted. Polyclonal rabbit anti-tPA diluted at 1:3000 (of 5 mg/mL) was incubated for 1 hour at room temperature. Alkaline-phosphatase conjugated with goat anti-rabbit IgG (1:15,000) was diluted in 1 %BSA TBS-T then introduced to the washed plate for 1 hour at room temperature. The colour was developed by addition of p-nitrophenyl phosphate substrate and incubation overnight at 4°C. The absorbance was taken at 405 nm by Softmax ELISA reader.

2.3.3. Chromozyme Assay for the detection of tPA

Chromozyme tPA was purchased from Roche Molecular Diagnostics, the recombinant human tPA standard was purchased from Cortex Biochem. All other materials were ordered from Sigma.

Chromozyme tPA (Roche Molecular Diagnostics, Indianapolis, IN) is a substrate that is cleaved by tPA to form a 4-nitranilide which is measured at 405 nm. The substrate solution was prepared as recommended by the manufacturer and allowed to react to the tPA samples in 96 well plates at 37°C for 30 minutes, prior to addition of citric acid (10 % w/v) for termination of the reaction. The samples were then read using Softmax Pro and concentrations of tPA were estimated by comparison to a standard curve of standard tPA. The samples were untreated prior to incubation with the substrate.

2.4. Western Blots

2.4.1. SDS-PAGE

SDS-PAGE was run according to the discontinuous buffer system of Laemmeli (Laemmli 1970). The gels were prepared using 7, 10 or 12 % polyacrylamide, depending on separation desired from the gel. The SDS gel preparation and separation of the proteins using the BioRad Apparatus was followed from instructions given by BioRad. The Gel was run for 45 minutes at 200 Volts or until the dye front ran off the gel. Electrical current was supplied by a BioRad power supply (Model 1000/500).

Pre-stained protein ladder was purchased from BioRad with a molecular weight range of 10 to 250 kDa. Typically, 5 μL of standard was loaded onto each SDS-PAGE well.

2.4.2. Determination of IFN-beta

The gel (Section 2.4.1) was then transferred to a nitrocellulose membrane following the procedure provided by BioRad. After transferring the gel to nitrocellulose the nitrocellulose was treated overnight with blocking buffer (3 % BSA/PBS) at 4°C. The membrane was then incubated with monoclonal anti IFN-beta (0.5 μg/mL) (Chemicon International) for 3 hours. The blot was incubated with alkaline phosphatase conjugated goat anti-mouse IgG for 2 hours. The substrate (Nitroblue tetrazolium (0.3 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/mL) in 5 mM $MgCl_2$ was then applied for up to 15 minutes for colour formation. The reaction was stopped by addition of PBS-EDTA, and the blots were rinsed thoroughly with distilled water.

In cases where densitometry analysis was required, a second western blot was detected using CDP-STAR (Disodium 2-chloro-5-(4-methoxyspiro [1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan]-4-yl) phenyl phosphate) (Roche, Bedford, MA). The use of CDP-STAR allowed for ultra-sensitive and rapid chemiluminescent detection of alkaline phosphatase activity (see 2.4.4.).

2.4.3. Determination of tPA

The same protocol was followed as for the IFN-beta western blots with the following changes: After blocking the blot overnight with blocking buffer (3 % BSA/PBS) at 4°C, the membrane was then incubated with polyclonal antibody against tPA (0.5μg/mL) (Cortex Biochem) for 3 hours. The blot was incubated with alkaline phosphatase conjugated goat anti-mouse IgG for 2 hours. The substrate (Nitroblue tetrazolium (0.3mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.15mg/mL) in 5mM $MgCl_2$ was then applied for up to 15 minutes for colour formation.

2.4.4. IFN-beta Densitometry Analysis

AlphaEaseFC™ software was used to scan and quantify IFN-beta bands following detection via Western Blot. The glycosylated and non-glycosylated forms of the IFN-beta were quantified in relation to total IFN-beta. Specific templates allowed for determination of pixelated areas of the western blots. AlphaEaseFC™ automatically detects and integrates the area under each peak and represents band intensity as a percentage of each peak contributing to a total density of 100 %.

2.5. IFN-beta Purification by Blue Sepharose 6 Fast Flow columns

2.5.1. Reagents and buffers

1st washing buffer: 0.2M phosphate and 0.15M sodium chloride with final pH of 7.2

2nd washing buffer: 0.2M phosphate and 2M sodium chloride with final pH of 7.2

Elution buffer: 0.2M phosphate, 2M sodium chloride, 50 % Ethylene glycol with final pH of 7.2

2.5.2. Procedure

The Blue Sepharose 6 Fast Flow column (GE Healthcare, Uppsala, Sweden) was washed (column size: 5 mL), prior to each purification, using 40 mL of 0.2M phosphate and 0.15M sodium chloride with a pH of 7.2. Filtered supernatant (200 mL) was loaded onto the column at a flow rate of 3 mL/min. After loading, the column was washed with 8 bed volumes of 1st washing buffer and 8 bed volumes of elution buffer. The IFN-beta was eluted with 8 bed volumes of elution buffer. The IFN-beta fraction was collected and stored at -20°C. Following elution the columns were washed with 8 bed volumes of 1st washing buffer and stored. Prior to use of the collected IFN-beta, the protein was dialysed against PBS with 2 % glycerol at 4°C.

2.5.3. SDS-PAGE gels

Glycosylated and non glycosylated IFN-beta species were separated by 12 % SDS-PAGE. The gels were stained using Coomassie Blue (0.2 % Coomassie Blue

7.5 % acetic acid, 50 % ethanol), and then destained overnight (in 0.75 % acetic acid, 10 % ethanol). The Coomassie stained bands were cut from the gels and frozen at -20°C until required for glycan extraction.

2.6. Oligosaccharide analysis

2.6.1. Release of N-glycans using PNGase F

N-glycans were released from IFN-beta by incubation with 50 µL of PNGaseF (peptide-N-glycosidase F) (Roche, Manheim, Germany) at an initial concentration of 1 U/mL in 1 mL of 20 mM $NaHCO_3$ buffer (pH 7.0). The gel bands were cut into small pieces (1 mm^2) then washed in 1 mL of 20 mM $NaHCO_3$ buffer (pH 7.0) for 60 minutes. The buffer was then removed and replaced with a 1mL of a 1:1 acetonitrile:20 mM $NaHCO_3$ buffer and allowed to incubate at room temperature for 30 minutes. Once again these two wash steps were repeated for 30 minutes each. The acetonitrile works to remove the Coomassie stain from the gel pieces. The gel was dried in a SpeedVac (Savant, Ramsey, USA) and 50 µl of PNGaseF was added to the dried gel. Additional 20 mM $NaHCO_3$ was added until the buffer level was high enough to cover the gel. The gels were then incubated at 37°C for 12 to 16 hours.

After incubation, the supernatant was extracted and retained. To further remove glycans still lodged in the gel, 300 µL of distilled water was added to the gel and the sample was sonicated for 30 minutes. The supernatants from the sonicated gels were added to the initial supernatant. The sonication step was repeated using 300 µL distilled water and 300 µL of acetonitrile, with the addition of the supernatant to the initial supernatant. The pooled supernatants were then incubated with 40 µL H+ activated AG-

50 for 5 minutes in order to desalt the glycans extracts. After centrifugation at 4500 xg for 5 minutes, the supernatant was filtered through a 0.45 μm Millipore syringe filter. The filtered supernatant was then dried completely in preparation for 2-AB labelling.

2.6.2. 2-AB (Aminobenzamide) labelling and GlycoClean S cartridge clean up

The procedure followed for 2-AB labelling was as that described by Bigge *et al.* (1995). The completely dried glycans were fluorescently labelled using 5 μL of a 1 M solution of sodium cyanoborohydride ($NaBH_3CN$) in 0.35 M 2-AB mixed in 30 % glacial acetic acid in DMSO and incubated at 65°C for 2 hours. After labelling, the excess 2-AB was removed using GlycoClean S Cartridges (ProZyme, San Leandro, USA).

The Cartridges were washed with distilled water, and then primed with acetonitrile. The sample of 2-AB labelled glycans are loaded onto the disk and allowed to soak for 15 minutes. After adsorption the excess 2-AB is removed by washing with 65 % acetonitrile in water. The glycans are then extracted from the disk by washing with water. The desorbed glycans in water are then dried down in a vacuum centrifuge.

2.6.3. HPLC Analysis of Glycans in Normal Phase (NP) column

The Glycan analysis was completed in a TSK-GEL Amide-80 column. The dimensions of the column were 250 mm by 4.6 mm with the column temperature was 30°C throughout the analysis. The gradient was set up as follows, where the buffers used were 50 mM Formic Acid adjusted to pH 4.4 with ammonia (A) and Acetonitrile (B).

Time (min)	%A	%B	Flow rate (mL/min)
0	20	80	0.4
152	58	42	0.4
155	100	0	0.4
160	100	0	1
165	100	0	1
170	20	80	1
185	20	80	1
188	20	80	0.4
200	20	80	0.4

The 2-AB label was detected at 330 nm excitation wavelength, 420 nm emission wavelength, with 1000 EUFS (Emission Units Full Scale) and a gain of 1. A sample of 100 µL was prepared in 80 % acetonitrile.

2.6.4. HPLC Analysis of Glycans in an Weak Anion Exchange (WAX) Column

The Glycan analysis was completed in a GlycoSep C column. The dimensions of the column were 250 mm by 4.6 mm with the column temperature at 25°C throughout the running of the sample. The Gradient was set up as follows, where the buffers used were 20 % Acetonitrile in Water (A) and 20 % Acetonitrile in 250 mM Ammonium Acetate pH 4.5 (B).

Time (min)	%A	%B	Flow rate (mL/min)
0	100	0	0.4
6	100	0	0.4
34	0	100	0.4
39	100	0	0.4
60	100	0	0.4

The 2-AB label was detected at 330 nm excitation wavelength, 420 nm emission wavelength, with 1000 EUFS and a gain of 1. A sample of 100 μL was prepared in water.

2.6.5. Exoglycosidase digestions

A 2AB labelled sample, already analysed by NP-HPLC was treated sequentially with exoglycosidase enzymes.

Enzyme digests were performed at 37°C for 24 hours in 100 mM citrate/phosphate buffer, pH 4.5, 0.2 mM zinc actetate, 0.15 M sodium chloride.

Conditions for the individual digests are as follows:

Clostridium perfringes Sialidase (Sigma): 1-2 U/mL

Bovine testes β-galactosidase (Oxford): 1-2 U/mL

Jack bean N-acetyl β-hexosaminidase (Prozyme): 10 U/mL

Bovine kidney fucosidase (Oxford): 1 U/mL.

The removal of sugars from the 2-AB labelled glycan can be monitored by a shift in the peaks using NP-HPLC, leading to different retention times. The running of standards give rise to the expected peak retention times.

2.6.6. Sialylation Index Calculation

The predominant glycans structures identified in the immunoglobulin samples were asialylated (S0), monosialylated (S1) and di-sialylated (S2). In order to compare the degree of terminal sialylation of the structures, a sialylation index (GI) was assigned to each glycan profile.

$$SI = \frac{S2 + 0.5 * S1}{S0+S1+S2}$$

2.7. Microcarrier cultures

2.7.1. Cytopore

2.7.1.1. Cytopore bead preparation

Cytopore 1 and 2 microcarriers were used for the growth of the CHO cells lines in 100 mL spinner flasks. The beads were prepared by a procedure based on that provided by the manufacturer (GE Healthcare, Uppsala, Sweden). The microcarriers were autoclaved for 25 minutes after hydration of 1 g for 10 minutes in 100 mL of PBS (pH 7.2) at room temperature. After autoclaving, the beads were allowed to settle and the PBS was extracted. The beads were washed with fresh media 5 times to dilute out the PBS, then resuspended in 100 mL of fresh media to yield a final concentration of 10 mg/mL. To prevent bead attachment, bottles used for the preparation of the microcarriers and

culture vessels were siliconized with Sigmacote (Sigma) following the manufacturer's instructions.

In spinner cultures containing Cytopore, after cell inoculation, the stir rate was held constant at 40 rpm for the duration of the culture. In Cytopore bioreactor cultures, after cell inoculation, the stir rate was held constant at 40 rpm for 2 hours, raised to 60 rpm for 2 hours, 100 rpm for 2 hours and finally 120 rpm for the duration of the culture. The stir rate was held at 120 rpm to ensure a homogenous mixture of Cytopore throughout the bioreactor volume. Cells were found to enter microcarriers after 2 hours of stirring at 40 rpm.

2.7.1.2. Cell enumeration from Cytopore microcarriers

Crystal violet staining of nuclei was done as described in Section 2.2.2.2. Cells from microcarriers were removed by aspiriation (25X) through a 25 gauge needle which allowed the recovery of stained nuclei which were counted in a haemocytometer. This procedure was sufficient to remove all nuclei from the microcarriers, which were routinely checked for residual nuclei by microscopic examination following staining.

Microcarriers were also observed using fluorescence microscopy (Zeiss Axioimager Z1 with 20X/0.8 plan-apochromatic objective) to follow the removal of nuclei from the microcarriers. Fluorescence micrographs of nuclei stained with TO-PRO-3 Iodide (Invitrogen) (Ex 640 nm/Em 690 nm) were superimposed over images of Cytopore autofluorescence (Ex 470 nm/ Em 525 nm).

2.7.1.3. Calculation of cell density within the microcarrier

The cell density within the microcarrier was determined from the cell count and the proportional volume of the microcarriers within the culture. The bead / weight ratio of Cytopore was obtained by Yokomizo *et al.* (2004) as 3400 per mg. The volume of each microcarrier was calculated as 7.24×10^{-6} cm^3/bead by the standard formula ($v = 4/3\pi r^3$) and the mean radius of 120μm. Thus at a microcarrier concentration of 2 mg/mL the proportional volume occupied by the beads was 5 % of the total culture volume.

2.7.2. Cytodex

Cytodex 1 and 3 were prepared in a similar manner to the Cytopore microcarriers. However the beads were allowed to swell in PBS for 3 hours at room temperature prior to autoclaving.

2.8. Metabolite Calculation

2.8.1. Glucose

The concentration of glucose was determined based on a colorimetric reaction caused by an enzymatic reaction. The kit utilized was the Glucose Trinder (Sigma, 315-100). The reactions involved are:

Reaction 1:

$$\text{Glucose} + H_20 + 0_2 \longrightarrow \text{Gluconic Acid} + H_2O_2$$

Reaction 2:

H_2O_2 + 4-Aminoatipyrine ⟶ Quinoneimine Dye + H_2O_2 + p-Hydroxybenzene Sulfonate

Quinoneimine Dye is detected at 505nm. The intensity of the color produced is directly proportional to the glucose concentration in the sample, and can be quantified by comparison to a standard glucose curve.

The media contains a glucose concentration of 4.5 g/L; thus a linear glucose standard curve was prepared between 0 and 5 g/L. The assay was run in 96-well plates, where 1 μL of standard or culture supernatant samples was added to each well in triplicate. Glucose Trinder (200 μL) was added to each well and incubated at 37°C for 15 minutes. The absorbance was determined with an automated Microplate Reader (BIO-TEK Instruments Inc.) at 490 nm.

2.8.2. Glutamine

A glutamine assay kit (Sigma GLN-2) was used to measure the glutamine concentration in the media. The assay is based on a reductive deamidation of L-glutamine by an enzymatic reaction (proprietary). Quantitation is accomplished by linking a dye directly to the reductive reaction. The reaction is specific for L-glutamine and does not cross-react with other amino acids or ammonia.

The assay was performed in duplicate in a 96-well plate. The media samples were compared to a standard curve ranging from 0.25 to 6 mM L-glutamine. The inclusion of an internal standard validated the lack of any inhibition or enhancement of the reaction due to media components other than L-glutamine. The internal standard was prepared by addition of 10 μL of the undiluted standard to a well containing 290 μL of the culture medium.

To each well 5 μL of reaction buffer, 30 μL of sample or standard, 50 μL 1X Diluent buffer (proprietary) and 15 μL enzyme preparation. The samples were incubated for 1 hour at 37°C and read at 550 nm in an automated Microplate Reader (BIO-TEK Instruments Inc.).

A linear regression analysis of the standard L-glutamine was performed and the slope of the curve was used to determine the uncorrected L-glutamine concentration of the samples. The components within the media that may cause adverse reactions were corrected by the use of an internal standard. The recovery value, that is used to correct the sample values, was calculated by:

Recovery value = D = (A-B)/C

Where:

A = [L-glutamine] in the sample with the internal standard (spiked sample)

B = [L-glutamine] in the sample without the internal standard

C = [L-glutamine] added as the internal standard (0.2 mM since 10 μL of the 6 mM was added to 290 μL)

D = Recovery value

2.9. PF68 as a protectant

2.9.1. Viscometric Assays

A variable gap cone and plate viscometer (MC1) fitted with an MK91 measuring cone was obtained from Paar Physica, New Jersey, USA. The viscometer had a spinning cone and a stationary plate on which the sample was placed. The gap between the cone and plate was adjustable. An optimum gap size was determined to be 0.025 mm. A larger gap caused the entrainment of bubbles while a smaller gap could lead to friction between the cone and the plate at certain points.

The temperature of the lower plate was controlled by circulating water. In most cases the viscometer was operated with a shear rate of 11,000 s^{-1} for 12 minutes applied to $2x10^6$ cells/mL at a temperature of 37°C with a gap distance of 0.025 mm between the cone and plate. After this treatment the viable cell count was determined from each sample and this enabled the calculation of the normalized cell viability (NCV) (Michaels *et al.* 1995).

$$NCV = \frac{\text{Final Viable Cell Count}}{\text{Initial Viable Cell Count}} \times 100\,\%$$

2.9.2. Cells grown in the presence of PF68 (Long term effects)

The cells were grown in T-75 T-flasks for 4 days under regular culture conditions (37°C and 10 % CO_2). Suspended cells were then decanted, supernatant removed then

added back to the flask with PBS-EDTA. The adherent cells were removed using trypsin, as described under section 2.2.1. The cells were then washed in media without PF68 and analysed for shear force resistance by viscometric analysis.

2.9.3. Cells treated to shear in the presence of PF68

Cells were treated to shear in the viscometer (as described in section 2.8.1) and 100 mL spinner flasks with rotation speeds of 100, 200, 300, 400, and 500 rpm. The spinner platform was calibrated using a strobe light where the rpm of the strobe light was set to a particular speed then the spinner platform was adjusted such that the stir bar appeared stationary.

2.9.4. Trypsinized cells treated to shear with and without PF68

Cells were treated with trypsin (0.5 % w/v) (Gibco) for variable time periods. Cells (2 x 10^6 cells/mL) in the exponential phase (day 4) were removed from suspension cultures. The cells were washed in PBS and pelleted by centrifugation. The cells were resuspended in a second wash of PBS, to which trypsin was added to a final concentration of 0.05 % w/v. After a fixed period of incubation, soybean trypsin inhibitor (0.09 % w/v; Sigma) was added to the trypsinized cells for 5 minutes. The cells were pelleted and resuspended in fresh media for counting using the trypan blue exclusion method (Section 2.2.2.1).

After the cells were treated with trypsin, cells were exposed to shear stress in the viscometer as described above.

2.10. The effect of trypsin on cells

2.10.1. The protection effect of PF68 on trypsinized cells

The protection effect of PF68 on trypsinized cells was assessed by damaging cells with trypsin as described in section 2.8.4. The damaged cells were then inoculated into 100 mL spinner flasks containing media with and without PF68. The cultures were incubated at 37°C for 4 days, with daily monitoring using the trypan blue viability method (Section 2.2.2.1).

2.11. Adherence studies

The percentage of cell adherence was determined for cells grown in T-flasks. The non-adherent suspension cells were decanted, centrifuged, washed with PBS-EDTA (1 mM) and resuspended in medium before counting. The T-flasks with the adherent cells were washed with PBS-EDTA (1 mM) and treated with trypsin (0.5 % w/v) for 3 minutes and then Gibco soybean trypsin inhibitor (0.09 % w/v) was added. Cells were resuspended in CHO-SFM and counted. The cell counts were converted to cells/mL in the original flask and the cell adherence was determined.

2.11.1.

$$\text{cell adherence} = \frac{\text{adherent cells}}{(\text{adherent} + \text{suspended cells})} \times 100\ \%$$

2.11.2.

2.11.3. PF68 Concentration dependent adherence

Cell adherence was determined, as described above (2.11), for cells grown in varying concentrations of PF68. The cells were inoculated into T-flasks containing varying concentrations of PF68 and grown for 4 day passages.

2.11.4. The removal of PF68 over passages

The removal of PF68 over passages was determined by growing cells in PF68-free media for 9 passages. At every three passages the cells were reintroduced into PF68 to test if the adherence effect was reversible by addition of PF68. The cells were grown in PF68 for three passages and the percentage of adherence was calculated as mentioned above (Section 2.11).

Section A – The use of Microporous and Macroporous Microcarriers for Recombinant Protein Production

Introduction

The increase of surface area in a culture can be induced by the incorporation of microcarriers into the culture. The microcarriers allow cells to grow in a pseudosuspension type culture where cells can attach to a positively charge bead (solid) or embed within the mesh (porous) and grow to high cell densities.

Microcarriers can be divided into two categories, microporous and macroporous. Microporous microcarriers have small pores on the surface, but beads are generally solid with the ability to grow cells on the surface of the bead. Macroporous microcarriers are porous, and enable cells to enter the microcarrier and grow to high cell densities.

The different microcarriers used in this study were Cytodex (microporous) and Cytopore (macroporous) microcarriers. Both microcarriers were used to test the effects of increasing cell to surface adhesion on the growth and productivity of IFN-beta and tPA producing CHO cells.

Chapter 3

3. The use of microporous microcarriers for CHO cell culture

3.1. Introduction

Microcarriers were originally developed as spheres capable of providing an enhanced substratum for anchorage-dependent cells in stirred cultures. Cytodex is the most widely used type of microcarrier and comprises a dextran matrix charged to a specific capacity with diethylaminoethyl groups (GE-Healthcare 2005). These are often referred to as "solid" microcarriers although in reality they are microporous, allowing nutrients but not cells to flow through the interior. Each microcarrier bead, with a diameter of around 180 μm, has the capacity to retain approximately 200 cells as a monolayer on the surface.

The use of Cytodex microcarriers has been limited to the growth of anchorage-dependent cell lines, as the cells must be able to attach to the surface of the microcarrier and grow attached to this surface. The cells can then be removed by treatment with a proteolytic enzyme such as trypsin, breaking the bond between cells and bead. There are 3 types of Cytodex that are available on the market; Cytodex 1, 2, and 3. Cytodex 1 and 2 have charges associated with the bead; type 1 has the charge dispersed throughout the bead whereas type 2 is charged solely on the surface of the bead and Cytodex 3 is collagen coated to increase surface attachment. Cytodex 1 and 3 are the most widely used Cytodex microcarriers (GE-Healthcare 2005).

In our cell system, although cells are able to grow in suspension, we tested the growth and productivity effects of the IFN-beta producing and tPA producing CHO cells on the Cytodex 1 and 3 microcarriers. CHO cells are naturally found adherent in cultures;

however with the use of serum free media the cells grow in suspension. Regardless the cells adhere to Cytodex microcarriers, due to the strong positive charge on the Cytodex 1 type and the collagen coating on the Cytodex 3 type.

3.2. Results

3.2.1. The growth of cells on Cytodex Microcarriers

The Cytodex microcarriers were used to increase the surface area with a spinner flask culture. Although the two CHO cell lines used in the study were adapted to grow in suspension, we decided to test the effect of Cytodex 1 and 3 microcarriers on the growth of these cell lines. The goal of the use of Cytodex in culture was to enhance productivity of the recombinant proteins, either by increasing cell yield, or creating an optimal situation for the cells whereby the cells can produce more recombinant protein.

Cytodex microcarriers are visualized in Figure 3.1. The cells were found to attach immediately to the surface of the microcarrier (A), but remained in a spheroid cell shape. Later, the cells appeared to flatten out and take on a more fibroblastic appearance (B). The first image is a representation of a day 1 culture (24 hours post-inoculation) compared to a day 4 image (96 hours post-inoculation).

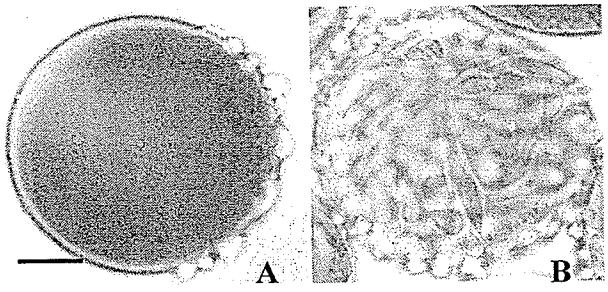

Figure 3.1: Photomicrographs of Cytodex 3 microcarriers with (A) day 1: a small cell population; (B) day 4: most cells were found to be adherent in the 3.0 mg/mL microcarrier culture. The microcarriers were stained with trypan blue to determine the cell viability on the Cytodex 3 microcarrier. The bar indicates 50 μm.

3.2.2. The effect of Cytodex 1 on cell growth

To determine the effect of the Cytodex microcarriers on the growth of the two CHO cell lines, we monitored the cell growth. Figure 3.2 shows the growth profiles of IFN-beta at tPA producing CHO cells on Cytodex 1 microcarriers. The cell counts are taken as nuclei counts to allow for comparisons between Cytodex and Cytopore cultures.

Figure 3.2A shows the growth of the IFN-beta producing cell line. The suspension culture is indicated by the dark circles, and reaches a maximum cell yield of $4.5x10^6$ cells/mL. It appears that the suspension culture has a higher specific growth rate during the initial period of the culture but the specific growth rate declines over time, presumably where the concentration of the nutrients available in the culture declines. It appears that at 5.0 mg/mL Cytodex 1, although having a lengthy lag phase, the cells grow to a high cell density towards the end of the culture yielding close to 4×10^6 cells/mL. However, it is important to note that the culture containing 3.0 mg/mL Cytodex 1 yield the maximum cell density of over $5x10^6$ cells/mL by day 7 of the culture.

At the beginning of the experiments, the microcarrier cultures appear to have no cells in suspension. Initial monitoring of the cell to bead attachment by microscopy showed the cells attaching to the beads within the first 3 to 4 hours of the culture. However, over time as the cell density increases the cells begin to detach from the microcarriers and grow in suspension (Figure 3.3). At the lowest microcarrier concentration of 1.0 mg/mL, there was the highest cell density in suspension, where less than $1x10^6$ cells/mL are found to grow in suspension at day 7 of the culture. The cells grown in 3.0 mg/mL and 5.0 mg/mL Cytodex 1 microcarrier cultures show a fraction of this population growing in suspension.

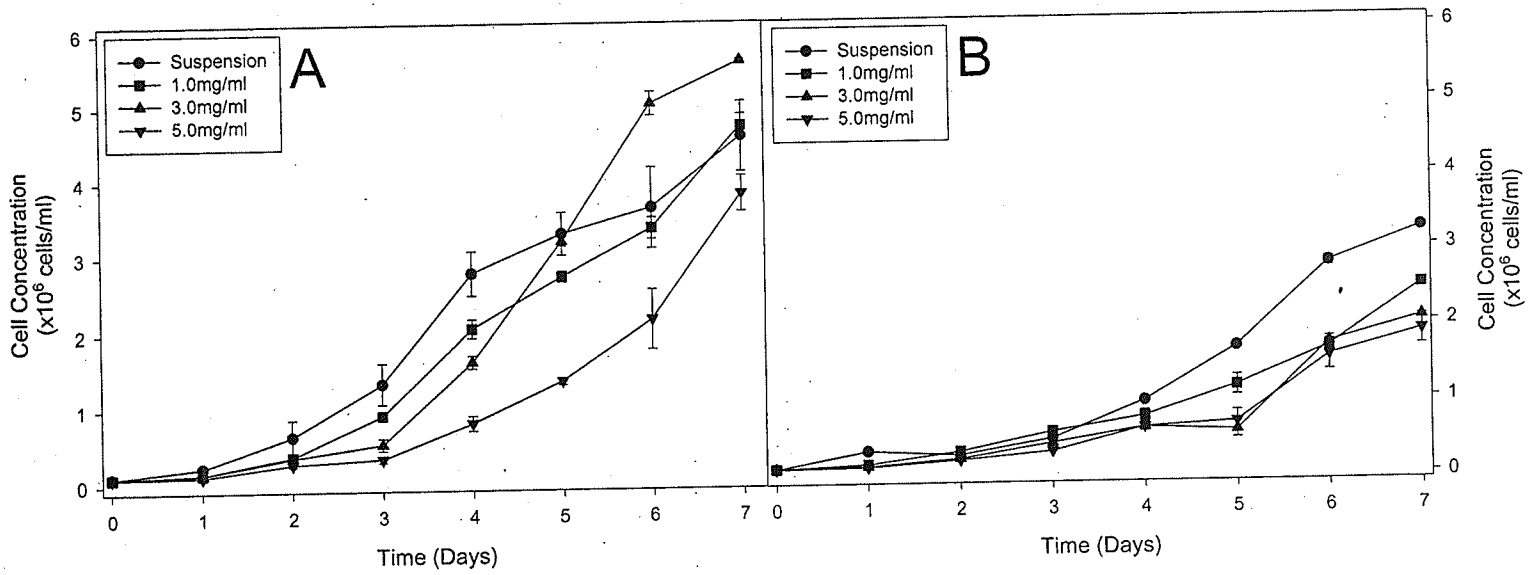

Figure 3.2: Growth profiles of IFN-beta producing (A) and tPA producing (B) CHO cells on Cytodex 1 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 1.0 mg/mL (■), 3.0 mg/mL (▲) and 5.0 mg/mL (◆) Cytodex 1 microcarriers or no microcarriers (●). All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Cell concentrations were determined by crystal violet staining for nuclei enumeration. The values are expressed as the cell densities within each microcarrier taking into account the proportional volumes occupied in each culture. Samples are means ± SEM between duplicate cultures.

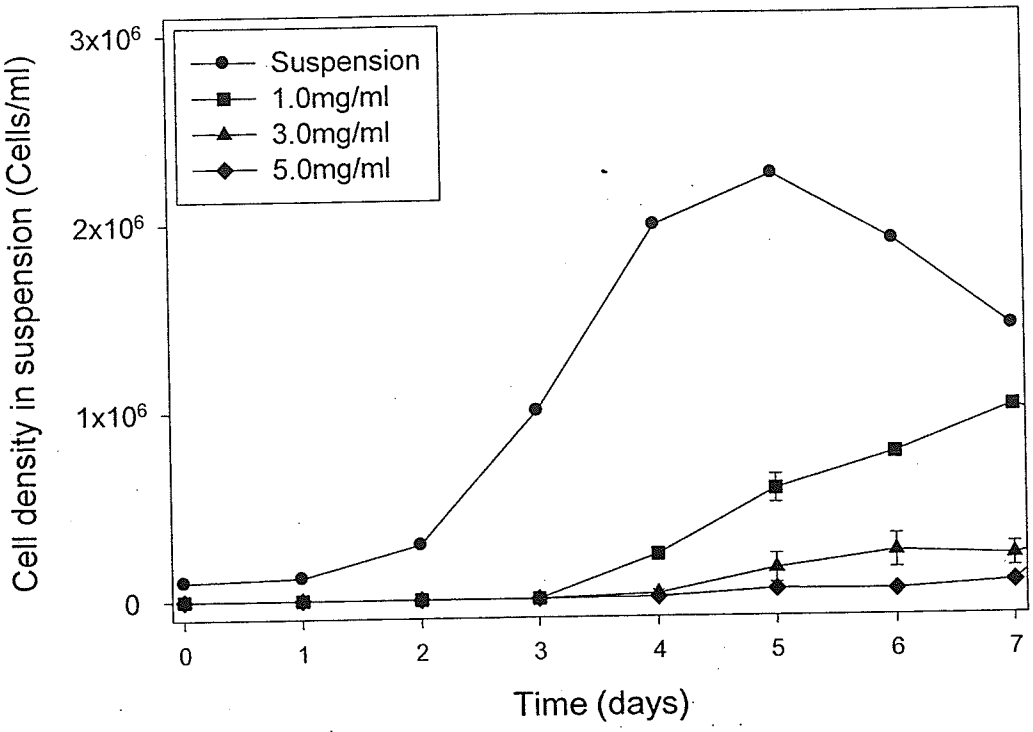

Figure 3.3: Cells in suspension for IFN-beta producing CHO cells on Cytodex 1 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 1.0 mg/mL (■), 3.0 mg/mL (▲) and 5.0 mg/mL (◆) Cytodex 1 microcarriers and suspension cultures (●). All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Cell concentrations were determined by the trypan blue exclusion method. Samples are means ± SEM between duplicate cultures.

A similar growth pattern was seen for the tPA cell line in Cytodex 1 microcarriers (Figure 3.2B). The Cytodex concentrations compared were 1.0 mg/mL, 3.0 mg/mL and 5.0 mg/mL. The tPA cell line appeared to grow to lower cell concentrations than the IFN-beta cell line. The maximum cell density obtained in any Cytodex 1 microcarrier culture was approximately $2.5x10^6$ cells/mL with 1.0 mg/mL Cytodex 1. The maximum cell density reached in the suspension culture was about $3.2x10^6$ cells/mL by day 7 of the

culture. The tPA cells were found to grow attached to the Cytodex 1 microcarriers, where there was an insignificant number of cells to grow in suspension by the end of the culture.

The confluency of the cells on the microcarrier often limits the growth of the cells. Figure 3.4 shows the cell to bead ratio of IFN-beta (A) and tPA (B) cell lines on Cytodex 1 microcarriers. In the case of IFN-beta in 1 mg/mL Cytodex 1, the maximum cell concentration per Cytodex microcarrier is approximately 1100 cells per bead. This maximum is reached at day 7. The cell concentrations of the 3 mg/mL and 5 mg/mL Cytodex 1 microcarrier cultures, in comparison, reach a fraction of the cell to bead ratio, where the 3 mg/mL and 5 mg/mL samples have a maximum ratio of 400 and 200 cells/bead, respectively, at day 7. This maximum density reached with the 1.0 mg/mL Cytodex explains why there are many cells found in suspension at this point of the culture. The cell distribution on the higher concentration of the microcarriers are not as confluent, and thus could tolerate a higher cell attachment and growth. However, looking at Figure 3.4, we can see that the cells are beginning to grow in suspension, and thus we could conclude that the Cytodex 1 microcarriers are not efficient for the growth of this CHO cell line.

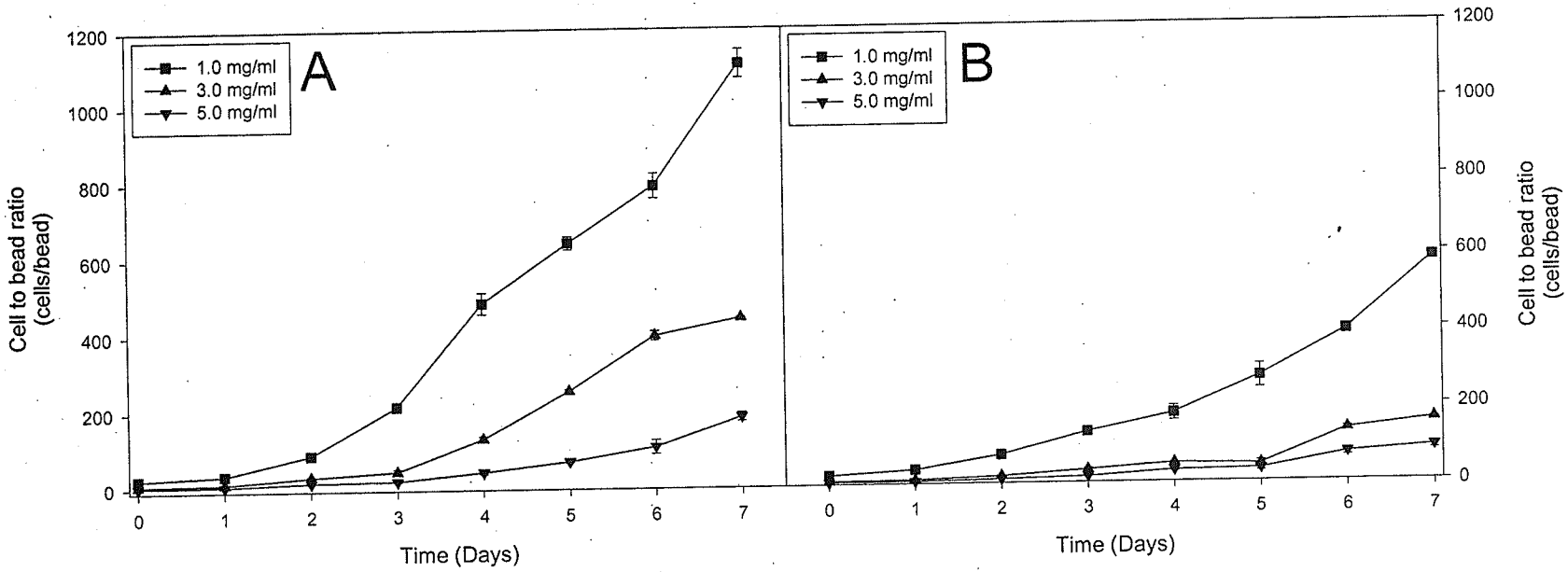

Figure 3.4: Nuclei to bead ratios for IFN-beta (A) and tPA (B) producing CHO cells on Cytodex 1 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 1.0 mg/mL (■), 3.0 mg/mL (▲) and 5.0 mg/mL (◆) Cytodex 1 microcarriers. All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Cell concentrations were determined by crystal violet staining for nuclei enumeration. The values are expressed as the cells attached to each microcarrier (calculated using values from Yokomizo *et al.*, 2004). Samples are means ± SEM between duplicate cultures.

The cell to bead ratios of the tPA producing CHO cells (Figure 3.3B) were quite different compared to those of the IFN-beta CHO cell line. The 1.0 mg/mL had a maximum ratio of 300 cells to beads, where the 3.0 mg/mL grew to a maximum cell to bead ratio of 150, and 5.0 mg/mL grew to just under 100. Comparing the growth patterns of the IFN-beta producing CHO cells and the tPA producing CHO cells on Cytodex 1 microcarriers allow us to see the growth differences of two different CHO cell lines. Although they originated from the same parental cell lines (CHO-K1), the transfections and the consequent selections that were assessed gives two different growth patterns of these CHO cells.

3.2.3. The effects of Cytodex 1 on IFN-beta and tPA production

The volumetric and specific production of IFN-beta derived from control (suspension) and Cytodex 1 microcarrier spinner cultures are as shown in Figure 3.5 where the left panel shows the native titers (A) and the right panel the denatured titers (B) (See Appendix A). The IFN-beta protein titers were equal to or less than that of the standard suspension cultures, thus there was no advantage in using the Cytodex microcarriers for the production of IFN-beta.

However, there appears to be a difference in the specific productivity when comparing the cells production during the exponential phase. The cells grown on the 1.0 mg/mL Cytodex microcarriers have almost double specific productivity at 2.6 pg/cell-day, compared to the suspension culture producing 1.2 pg/cell-day.

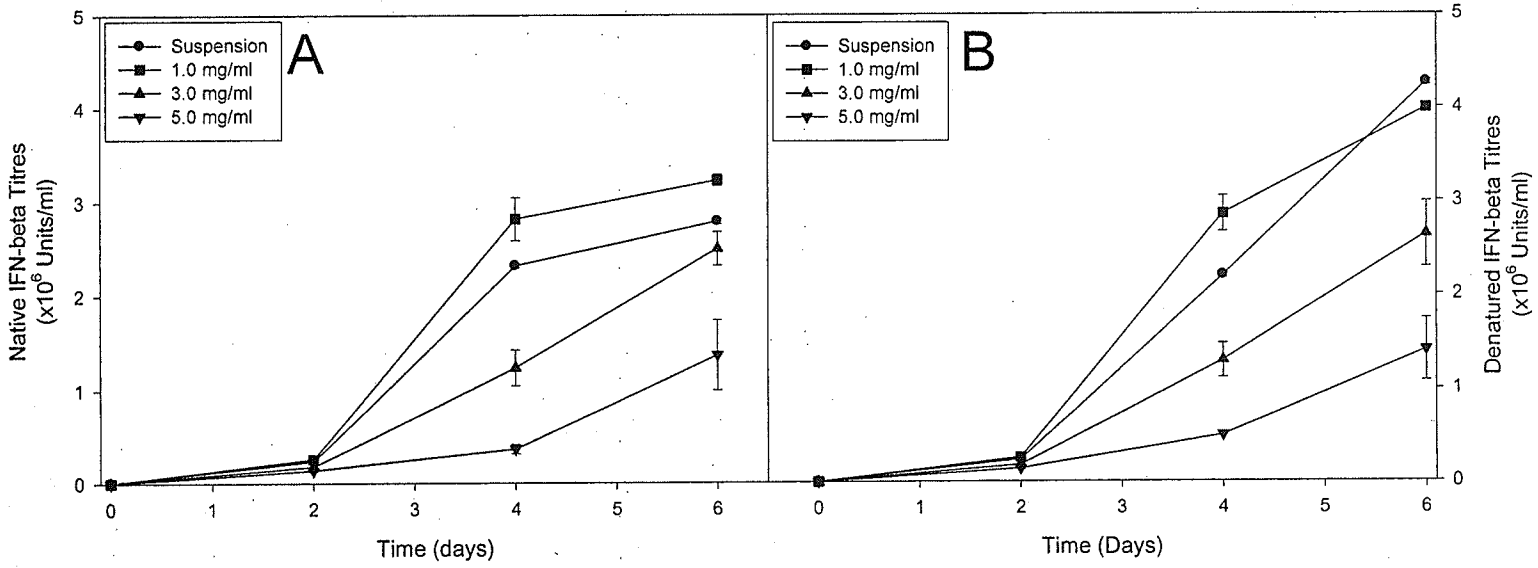

Figure 3.5: Maximum volumetric production of IFN-beta from CHO cells grown in Cytodex 1 microcarrier cultures in 100mL spinner flasks. All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Protein was quantified using ELISAs. Samples were quantified by native protein yield (A) and denatured (B). Samples are means ± SEM between duplicate cultures.

The aggregation of IFN-beta must be monitored to ensure efficient production (Refer to Section 2.3.1). Figure 3.6 shows the aggregation of IFN-beta at day 6 of the culture. The aggregation of IFN-beta appears to decrease with the introduction of Cytodex 1 microcarriers in the culture, where the suspension culture had a maximum aggregation coefficient of 34 %. The cultures with Cytodex had decreased aggregation, where 1mg/mL Cytodex led to an aggregation coefficient of 29 % and the higher concentrations resulted in less than 10 % aggregation. However these lower aggregation coefficients were offset with decreased overall protein concentrations.

The volumetric production of tPA in Cytodex microcarrier cultures appears to be stimulated (Figure 3.7). The suspension culture had a maximum titre of 3100 Units/mL at day 5. However, from day 5 to day 7, the tPA titre decreases from roughly 3100 units/mL to 2500 units/mL. This decrease is well documented as a degradation reaction that occurs in the culture (Lin *et al.* 1993). Although there is a degradation occurring in the suspension culture, there does not appear to be degradation occurring in the 3.0 mg/mL concentration of the Cytodex 1 microcarrier cultures. The maximum product titers is attained at the highest concentration of Cytodex 1 tested (5.0 mg/mL) where there is a maximum of 5094 units/mL, reached at day 7. However, at day 6, the titre drops to 5021 units/mL, thus indicating that there is slowing down in the production of tPA.

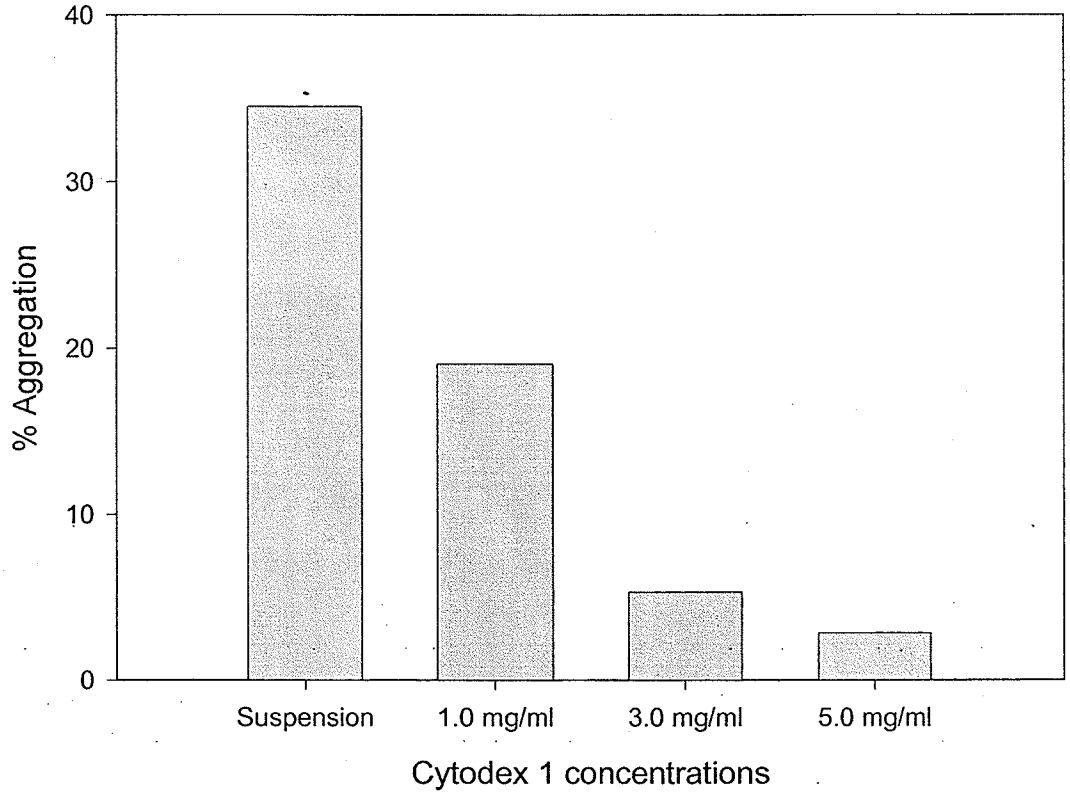

Figure 3.6: Aggregation of IFN-beta produced in Cytodex 1 microcarrier cultures at day 6. The aggregation was quantified by comparing the native protein yield and denatured protein yield in the supernatant protein sample. Samples are means between duplicate cultures.

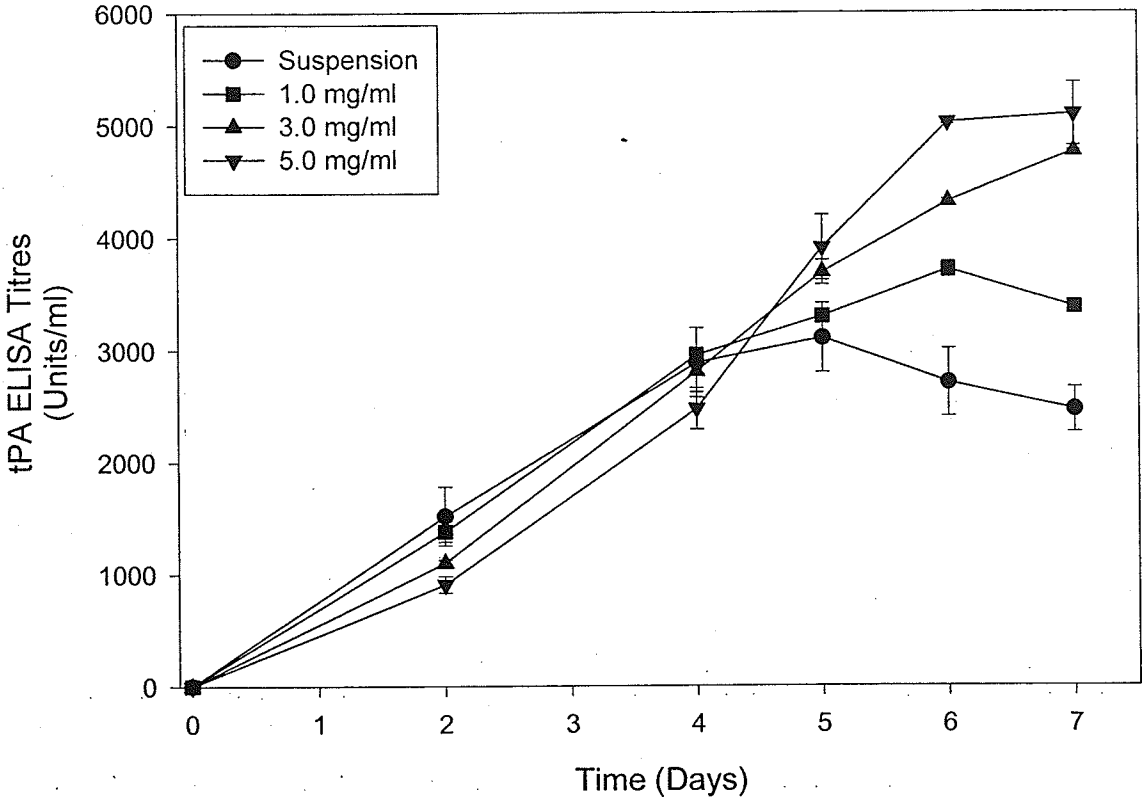

Figure 3.7: Maximum volumetric productivity of tPA from CHO cells grown in Cytodex microcarrier cultures in 100mL spinner flasks by ELISA. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.5mg/mL (▼), 1.0 mg/mL (■), 3.0 mg/mL (▲) and 5.0 mg/mL (◆) Cytodex 1 microcarriers and suspension cultures (●). Samples are means ± SEM between duplicate cultures.

3.2.4. The effect of Cytodex 3 on CHO cell growth

Cytodex 3 is a collagen coated microcarrier, and encourages the cell to bead attachment via protein-protein interactions. We observed a similar growth pattern with the IFN-beta producing CHO cells on Cytodex 3 microcarriers compared to cells grown on Cytodex 1 microcarriers (Figure 3.8A). The cell yields of the IFN-beta producing cells in suspension reached by the end of the 7 days were less than the cell yields of the Cytodex 1 cultures. However, the cells appeared to have a lengthened lag phase, thus leading to a lower cell population initially. This lengthened lag phase can be due to a strengthened cell attachment due to the collagen coating that may cause cells to attach and spread onto the surface of the Cytodex 3 microcarriers.

The 3.0 mg/mL Cytodex 3 concentration yielded a maximum cell count of 4.1×10^6 cells/mL at day 7. However, the growth rate of the cells at each respective Cytodex 3 concentration was consistent throughout the culture period.

The cell to bead ratios for the IFN-beta producing CHO cells were similar for Cytodex 3 as Cytodex 1 microcarriers (Figure 3.9A). The maximum cell number per bead for those cells grown in 1 mg/mL Cytodex 1 was about 1100 cells. Where cells grown on 3.0 mg/mL and 5.0 mg/mL grew to just under 500 cells/bead and 200 cells/bead, respectively. Another concentration was also tested, 0.5 mg/mL, where the intention was to yield a high cell to bead ratio early on in the culture. The maximum cell yield per bead was around 2000 cells/bead. However, later in the culture there was a plateau of 1500 to 1600 cells/bead. This resulted in cells detaching from the microcarriers early on in the culture.

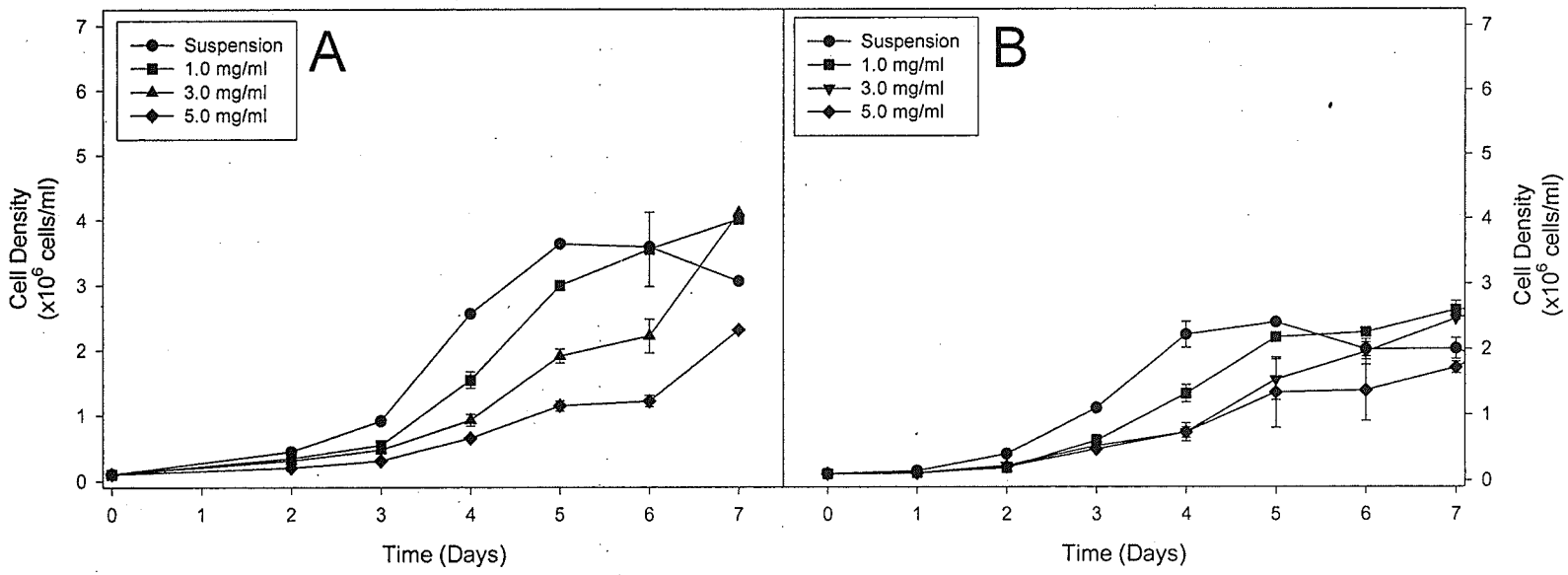

Figure 3.8: Growth profiles of IFN-beta (A) and tPA (B) producing CHO cells on Cytodex 3 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.5mg/mL (▼), 1.0 mg/mL (■), 3.0 mg/mL (▲) and 5.0 mg/mL (◆) Cytodex 3 microcarriers or no microcarriers (●). All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Cell concentrations were determined by crystal violet staining for nuclei enumeration. The values are expressed as the cell densities within each microcarrier taking into account the proportional volumes occupied in each culture. Samples are means ± SEM between duplicate cultures.

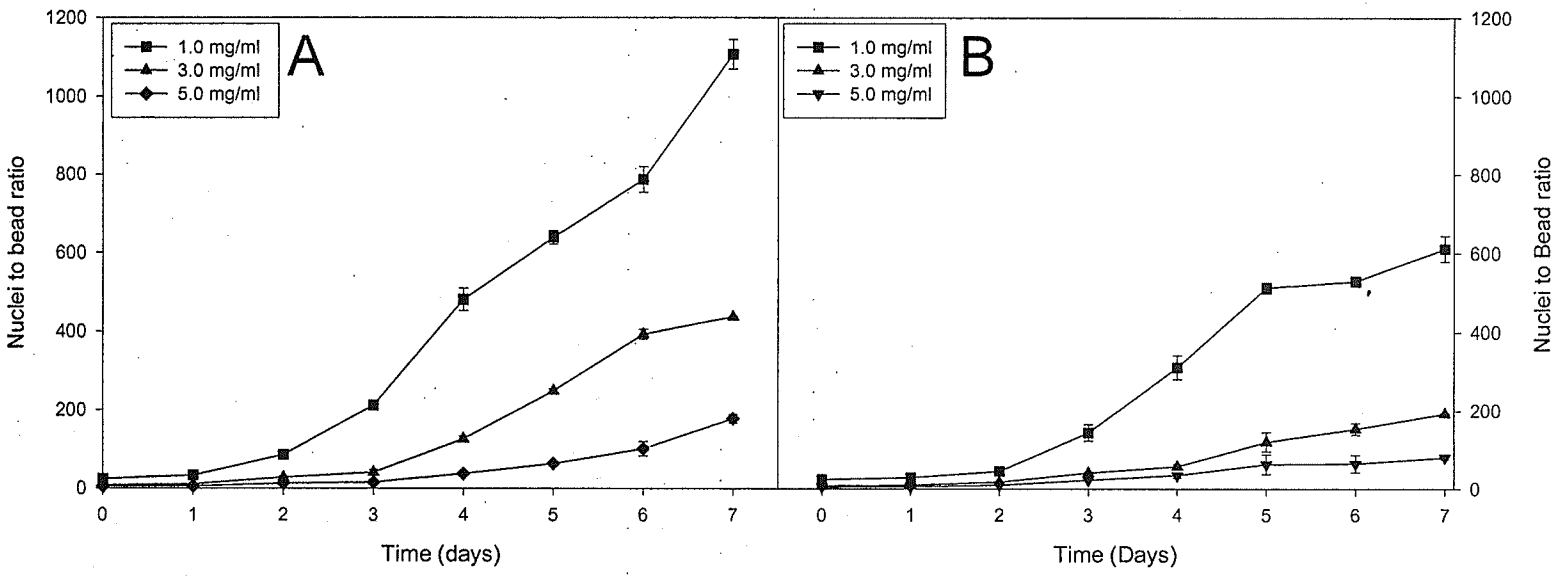

Figure 3.9: Nuclei to bead ration for IFN-beta (A) and tPA (B) producing CHO cells on Cytodex 3 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.5mg/mL (▼), 1.0 mg/mL (■), 3.0 mg/mL (▲) and 5.0 mg/mL (◆) Cytodex 3 microcarriers. All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Cell concentrations were determined by crystal violet staining for nuclei enumeration. The values are expressed as the cells attached to each microcarrier (calculated using values from Yokomizo *et al.*, 2004). Samples are means ± SEM between duplicate cultures.

At inoculation, all cells immediately attached to the Cytodex 3 microcarriers. However, after days 3 and 4, the cells started to detach and grow in suspension (Figure 3.10). The lower concentration (1.0 mg/mL Cytodex 3) culture had over $1x10^6$ cells/mL in suspension by the end of the culture, whereby essentially 25 % of cells were found in suspension. At the higher concentrations of Cytodex 3, the cell population found in suspension was 10 % or less.

The tPA cell line once again grew too much lower concentrations when compared to the IFN-beta cell line (Figure 3.8B). The maximum cell yield was $2.6x10^6$ cells/mL at day 7 in the 1.0 mg/mL Cytodex 3 concentration. However, the other Cytodex concentrations induced similar cell yields as the 1.0 mg/mL concentration by the end of the culture ($2.46x10^6$ cells/mL and $1.72x10^6$ cells/mL, in 3.0 mg/mL and 5.0 mg/mL, respectively). By the end of the culture period, there was an insignificant number of tPA producing cells found to grow in suspension in the Cytodex 3 cultures (data not shown).

The cell to bead ratio (Figure 3.9B) was much lower for the tPA cell line compared to the IFN-beta producing cell line, where the maximum cell to bead ratio was 610 cells/Cytodex 3 microcarrier. This ratio was attained in the 1.0 mg/mL Cytodex 3 microcarrier culture at day 7. However, in the 1.0 mg/mL Cytodex 3 culture the cell to bead coverage increased linearly from days 2 to 5, from 45 cells/bead to 510 cells/bead. Then over the last 2 days of the 1.0 mg/mL Cytodex 3 culture, the cells/bead increased by 100. The 3.0 mg/mL Cytodex 3 culture resulted in a maximum of 200 cells/bead by the end of a 7 day culture and 5.0 mg/mL Cytodex 3 cultures resulted in half of that level (100 cells/bead) at the end of the 7 days.

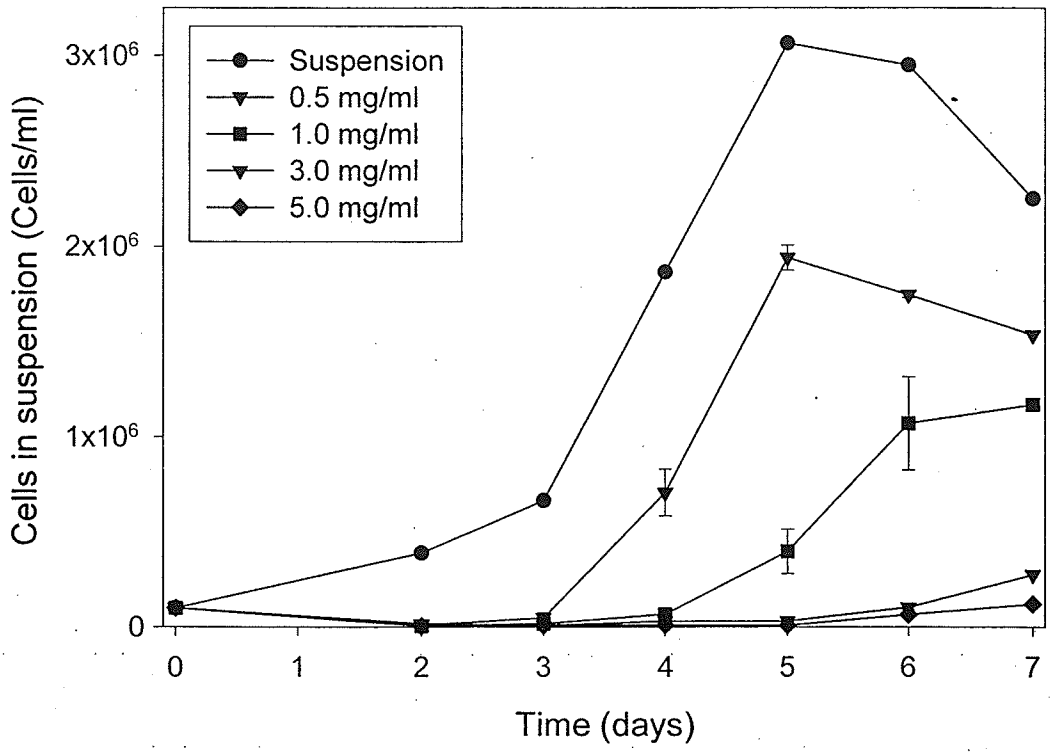

Figure 3.10: Cells in suspension for IFN-beta producing CHO cells on Cytodex 3 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.5 mg/mL (▼), 1.0 mg/mL (■), 3.0 mg/mL (▲) and 5.0 mg/mL (◆) Cytodex 1 microcarriers and suspension cultures (●). All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Cell concentrations were determined by crystal violet staining for nuclei enumeration. The values are expressed as the cells attached to each microcarrier (calculated using values from Yokomizo *et al.*, 2004). Samples are means ± SEM between duplicate cultures.

3.2.5. The effects of Cytodex 3 on IFN-beta and tPA production

The IFN-beta produced (Figure 3.11) in Cytodex 3 microcarrier cultures was not enhanced when compared to the suspension control cultures. The native protein (Figure 3.11A) yields ranged from $1.30x10^6$ units/mL to $3.42x10^6$ units/mL, with the suspension culture yielding $2.79x10^6$ units/mL while the denatured titers (Figure 3.11B) ranged from $1.75x10^6$ to $4.26x10^6$ units/mL. The aggregation of IFN-beta (Figure 3.12) of the suspension culture at day 6 was the greatest at 34 % compared to the cultures with Cytodex. The lower concentrations of Cytodex 3 induced lower aggregation of the protein product where 5mg/mL Cytodex 3 induced higher aggregation of 25 %.

Although the production of tPA appeared to be enhanced with the presence of Cytodex 3, (Figure 3.13) the suspension culture yielded a maximum titre of 3866 units/mL. The 5.0 mg/mL Cytodex 3 culture yielded a maximum titre of 5958 units/mL at day 7 of the culture. The lower concentration (1mg/mL) of Cytodex 3 yielded a maximum of 5006 Units/mL at day 4 of the culture, and then declined to 4325 units/mL at day 7.

3.2.6. Comparing Cytodex 1 and Cytodex 3

The protein yields of the CHO cells producing IFN-beta and tPA in Cytodex 1 and 3 microcarriers are as summarized in Table 3.1. The protein titers were normalized against the suspension culture to allow for comparisons between samples as well as the different proteins, where the suspension cultures were termed 1 Units/mL. This normalization

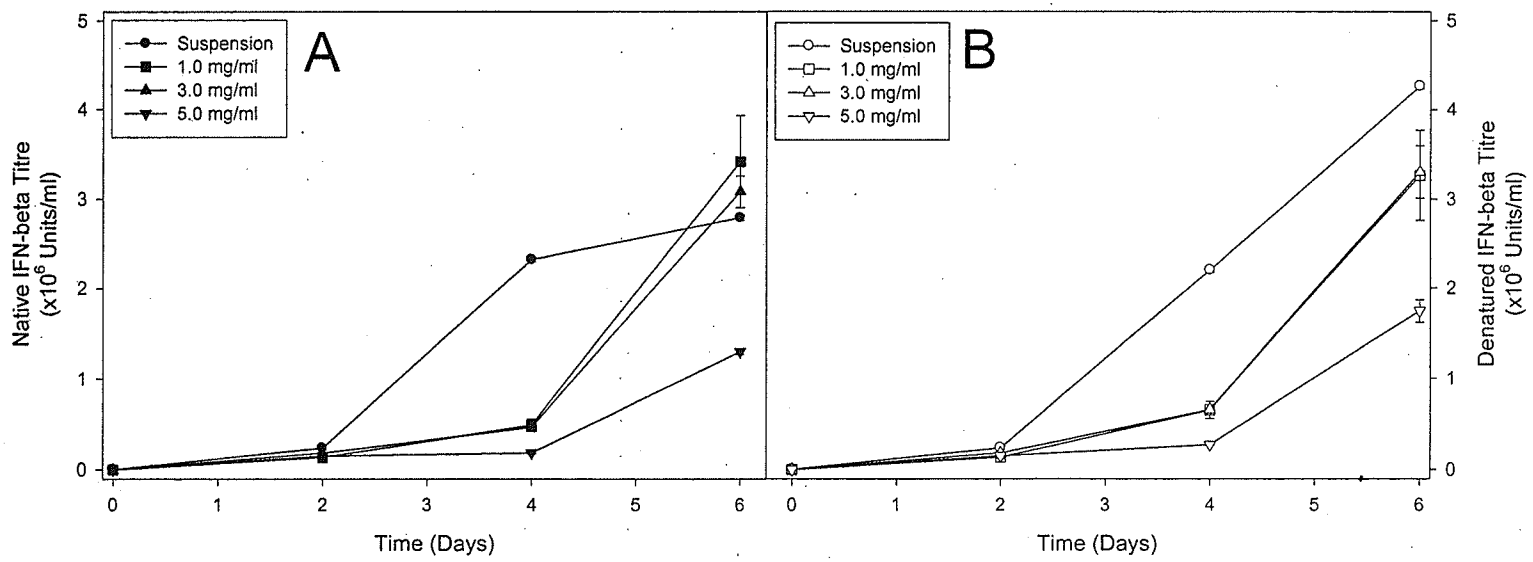

Figure 3.11: Maximum volumetric productivity of native (A) and denatured (B) IFN-beta from CHO cells grown in Cytodex 3 microcarrier cultures in 100mL spinner flasks. All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Protein was quantified using ELISAs. Samples are means ± SEM between duplicate cultures.

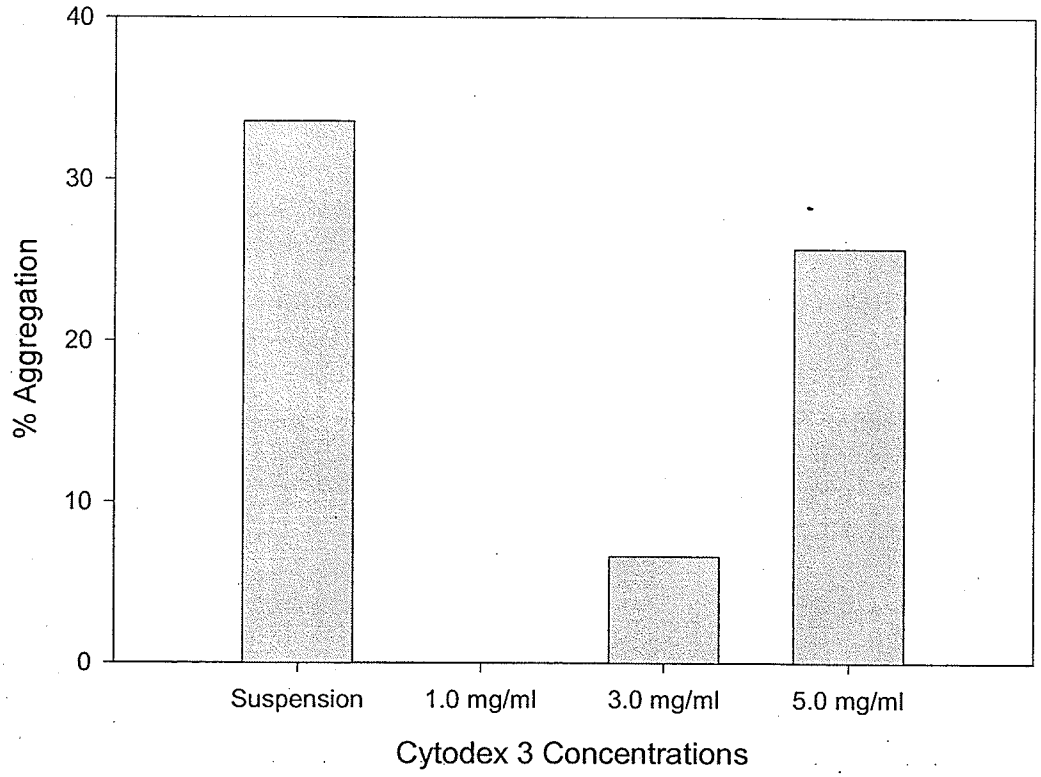

Figure 3.12: Aggregation in Cytodex 3 microcarrier cultures at day 6. The aggregation was quantified by comparing the native protein yield and denatured protein yield in the supernatant protein sample. Samples are means between duplicate cultures.

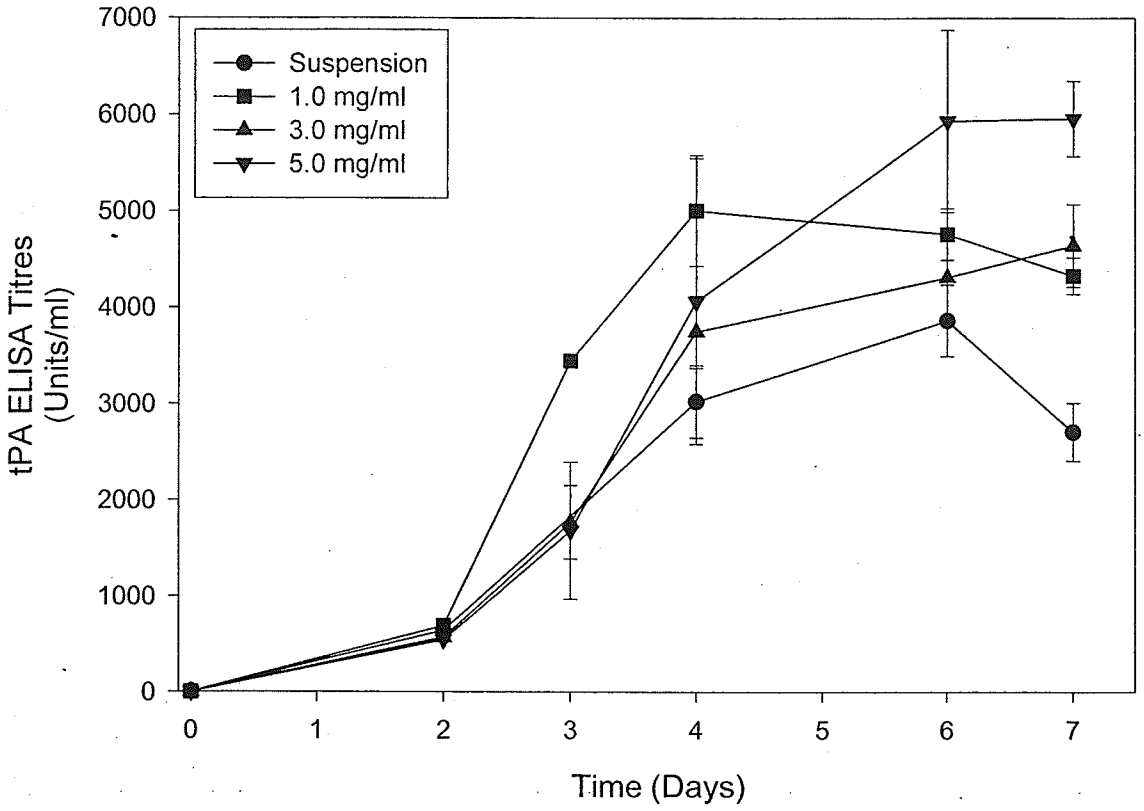

Figure 3.13: Maximum volumetric productivity of tPA from CHO cells grown in Cytodex 3 microcarrier cultures in 100 mL spinner flasks. All cultures were maintained in spinner flasks at 37°C with an overlay of 5 % CO_2 and with an impeller rotation speed of 40 rpm. Protein was quantified using an ELISA. Samples are means ± SEM between duplicate cultures.

Table 3.1: Summary table of the productivity of IFN-beta and tPA producing CHO cell lines in Cytodex 1 and 3 microcarrier cultures in 100 mL spinner flasks. Protein was quantified using an ELISA. IFN-beta samples were quantified by native protein yield (non-denatured) and denatured (total protein). The percent aggregation was determined by the ratio of aggregated protein to total protein evident in the supernatant sample. tPA samples were quantified by ELISA of supernatant samples.

Cytodex	Microcarrier Conc.(mg/mL)	Native IFN-beta titre (normalized units/mL)	Denatured IFN-beta titre (normalized units/mL)
1	Suspension	1.00	1.53
1	1.0	1.15	1.43
1	3.0	0.90	0.95
1	5.0	0.49	0.51
3	Suspension	1	1.51
3	1.0	1.28	1.22
3	3.0	1.15	1.23
3	5.0	0.49	0.65
Cytodex	Microcarrier Conc.(mg/mL)	tPA titre (normalized units/mL)	
1	Suspension	1.00	
1	1.0	1.20	
1	3.0	1.53	
1	5.0	1.64	
3	Suspension	1.00	
3	1.0	1.40	
3	3.0	1.30	
3	5.0	1.67	

would enable a direct comparison between Cytodex 1 and 3 titers as well as the difference in IFN-beta and tPA titers.

Monitoring the growth of the cells in different cultures is important; however it is essential to evaluate production of the recombinant proteins. Comparing the titers of IFN-beta on Cytodex 1 and 3 microcarriers, there seems to have been a decrease in protein titers with an increase in Cytodex 1 and 3 microcarrier concentrations. This trend was not consistent across both cell lines, as the tPA cell line showed higher titers with increased microcarrier concentrations. The IFN-beta production decreased with an increase in Cytodex from 1.0 mg/mL to 5.0 mg/mL, while the tPA titers increased with an increase in Cytodex from 1.0 mg/mL to 5.0 mg/mL. Thus the use of Cytodex microcarriers did not allow for similar production between both cell lines.

3.3. Discussion

Cytodex 1 and Cytodex 3 are microporous microcarriers that provide a surface for cells to attach and grow to high cell densities. Although CHO cells have been adapted to grow in suspension, and do not require a surface to which they could attach, they have been found to grow attached when inoculated into Cytodex cultures.

In terms of growth, there appears to be minimal benefits of the use of Cytodex in microcarrier cultures in the presence of some concentrations of Cytodex 1 and 3. At the end of the cultures (day 7) the 1.0 mg/mL and 3.0 mg/mL Cytodex 1 or Cytodex 3 concentrations often gave rise to higher cell concentrations than the suspension culture. However, this was not true for the tPA cell line grown on Cytodex 1 microcarriers, where

there was a 25 % decrease in the cell density of the 1.0 mg/mL culture compared to the suspension culture at day 7.

The growth of CHO cells on the Cytodex microcarriers was comparable to the growth of CHO cells producing Von Willebrand recombinant protein in batch culture by Mignot *et al.* (1990). Mignot *et al.* (1990) reported that the cells grew to a density of $3x10^6$ cells/mL up to day 12. The maximum cell loading was 250 cells/bead at day 12; however at day 6, up to 40 % of cells were in suspension. The differences could be attributed to the need for a substrate for attachment. As even comparing the IFN-beta and tPA producing cell lines, there appeared to be differences between the cells ability to attach and their differences in growth.

Wu *et al.* (2004) found that Vero cells growing at 37°C at a high Cytodex concentration of 10 mg/mL had high cell growth compared to that in lower concentrations of 2 mg/mL and 5 mg/mL. Kallel *et al.* (2003) reported that increasing the Cytodex 3 concentration from 3 to 4 mg/mL increased the cell yield; however comparing the cell to surface area they found that the surface area was limiting in the lower concentration of Cytodex 3 microcarrier culture. In this study, we reported that the lower concentrations of Cytodex microcarriers yielded the maximum cell concentrations.

Although the seeding efficiency was quite high and thus the percent of beads covered were high, there appeared to be cells in suspension early in the culture after day 3. The CHO cells appeared to fall off the Cytodex microcarriers early on in the culture, and thus the Cytodex microcarrier was not an efficient system for cell immobilization. After about day 3 to 5, depending on the microcarrier concentration, the cells that were attached were

found to grow in suspension. The cells did not have a permanent attachment, as agitation of the sample led to a high density of trypan blue stained cells in solution.

In most cases the use of Cytodex has been limited to the growth of adherent cell lines. One most common use of Cytodex is with Vero cells. Ng *et al.* (1996) reported the attachment rate of Vero cells onto Cytodex microcarriers was much greater than the attachment of Vero cells onto Cultispher-G (9.05×10^{-2} min^{-1} and 0.6×10^{-2} min^{-1}, respectively). This high attachment rate could be then used to explain the 10-fold higher cell density reached within a Cytodex 1 bioreactor culture compared to the cell density reached within T-flasks where cell density reached within the bioreactor was 5.6×10^{9} cells compared to 4.7×10^{7} cells in the T-flask (Sun *et al.* 2004). The cell density attained within the Cytodex cultures, in our case, was similar to the cells density attained in T-flask cultures where the range of cell density in a T-flask after 4 days of growth ranges between 2 and 3 $\times 10^{6}$ cells/mL. Although a direct comparison is not possible, comparing the cell numbers from our T-flask cultures and spinner flask cultures, we achieve up to 4 % of the cells in the T-flask compared to the spinner culture. However, the data presented by Sun *et al.* (2004) the cell numbers attained in T-flasks corresponds to less than 1 % of the population of the bioreactor culture. The discrepancy can be attributed to the difference in cell types, as Vero cells are anchorage dependent, where as CHO cells are not. As well, the conditions within a bioreactor are optimized and thus the bioreactor cultures are able to live longer further than T-flask or spinner flask cultures.

The increase in the specific growth rates of recombinant CHO cells producing human gamma-glutamyl transferase (Chevalot *et al.* 1994) from $0.025 h^{-1}$ in aggregated cells or

individual cells to $0.040h^{-1}$ in cells on Cytodex 1 microcarriers. Thus it was concluded that when the cells were propagated on solid supports the cells were able to grow faster than cells in suspension. This was argued to be due to the change in the morphological shape, where cells in suspension grow in a spherical shape in comparison to cells on solid supports which spread on the microcarrier support.

The use of microcarriers has not been limited to the growth of mammalian cell lines; insect cells have also been tested for optimal growth. However, studies found that an insect cell line, Lepidopteran (High-five and Sf-9) usually grown in suspension had poor growth results in the presence of Cytodex 1 microcarriers (Ikonomou *et al.* 2002). Liu and Wu (2004) found that a mosquito cell line, *A. albopictu* (C6/36), grew optimally in the presence of the Cytodex microcarriers when compared to a suspension culture.

Cytodex allows for the growth of many cells requiring attachment in a large scale growth capacity. These include VERO cells (Frazzati-Gallina *et al.* 2001; Liu and Wu 2004; Sun *et al.* 2004; Wu *et al.* 1998; Wu *et al.* 2004) C6/36 mosquito cells (Liu and Wu 2004), HeLa cells (Bleckwenn *et al.* 2005) and HEK 293 cells (Wu *et al.* 2002). These cell lines require a substratum on which the cells are able to attach and grow to high cell density, thus giving a higher surface area for virus penetration during inoculation. Cytopore and Cytodex are also used for the growth of insect cells. Ikonomou *et al.* (2002) looked at the growth of Sf-9 and High-Five cells on Cytodex, Cytopore 1 and 2, Cultispher G, and Fibra-Cel disks. They found that the cells did not grow attached to the Cytodex and that the cell density reached on Cytopore microcarriers was not high.

The use of Cytodex had usually been limited to the growth of adherent cell lines that are infected to produce viral vectors. However, there have been studies that looked at the microcarrier as a possibility for the growth of suspension cells that produce recombinant proteins. In particular, Chevalot *et al.* (1994) found that Cytodex 1 increased cell counts and thus resulted in a higher specific and volumetric productivity of the human gamma glutamyl transferase (GGT) protein produced by CHO cells in comparison to a suspension culture.

The productivity of the IFN-beta and tPA producing CHO cell lines were comparable to suspension cultures, and in some conditions the suspension culture was found to have higher titers than the microporous microcarrier culture. However, in the case of tPA, although titers from this cell line are generally low, under all conditions, the titers obtained from Cytodex 3 cultures were found to be slightly enhanced compared to a suspension culture. This shows the benefits of the cell attachment to the microcarrier surface. This enhancement was not witnessed in Cytodex 1 microcarrier cultures, and thus could be explained by the collagen coat on Cytodex 3 that would work to enhance cell attachment.

In our study, although the use of Cytodex for cell culture does not significantly enhance recombinant protein production from either cell line, there have been isolated papers reporting the enhanced production of recombinant proteins using this microcarrier system. Kong *et al.* (1999b) report that volumetric product from Cytodex microcarriers were slightly higher than equivalent cultures in other microcarriers such as Cultispher, Collagen and Biosilon. Chevalot *et al.* (1994) reported a significant increase in human

gamma-glutamyl transferase from CHO cells grown on Cytodex 1 microcarriers compared to aggregated or individually suspended cells. Thus it appears that the efficiency of the microcarrier system utilized is cell line dependent. In my study I used two separate CHO cell lines that grew and produced differently.

The use of Cytodex for these two CHO cell lines was not an efficient system for enhancement of protein production. Comparing the growth and productivity of the two CHO cell lines in cultures containing Cytodex microcarriers allowed us to see the differences in the cell lines. The tPA cell line showed slightly elevated productivities in the presence of the Cytodex 3 compared to the IFN-beta cell line, where under most concentrations of Cytodex there was no increase in productivity. However, the use of Cytodex microcarriers to culture the IFN-beta and tPA producing CHO cell lines was not considered to be an efficient system compared to suspension cultures for product maximization and thus was not further pursued.

Chapter 4

4. Comparison of Cytopore for Growth and Volumetric Productivity

4.1. Introduction

Macroporous microcarriers were designed to offer internal spaces where cells may be anchored or embedded and are able to grow to high cell densities in a pseudo-suspension culture. The entrapped cells within the carriers can be protected from high shear forces. The macroporous microcarriers can allow higher cell to bead loadings compared to the conventional microcarriers. This allows the microcarrier to reach high cell densities without being compromised by high spinner rotation speeds (Ng *et al.* 1996). There are various types of macroporous microcarriers that have been tested in the past. Of the many examples, Cytopore is a charged cross-linked cellulose microcarrier suitable for stirred-tank cultures (GE-Healthcare 2002). The average diameter of pore openings is 30 μm with the complete microcarrier having a diameter of up to 300 μm. There are two varieties of Cytopore – 1 and 2, which differ in their charge density. Cytopore 1 has a charge density of 1.1 meq/g, while Cytopore 2 has a charge density of 1.8 meq/g.

Macroporous microcarriers such as Cytopore offer internal pores for which cells can embed and grow to high cell densities. These high cell densities offer protection from high shear forces, especially in the case of sparging in bioreactors (Duerrschmid *et al.* 2003; Xiao *et al.* 1999). Cytopore microcarriers can be used for culturing adherent cell lines as well as cells adapted to grow in suspension. The cells are able to enter within the pores and grow to high densities.

In this study, the use of two genetically-engineered CHO cell lines were used as models to investigate the productivity of cells secreting human recombinant proteins that were grown to high densities within two types of macroporous microcarriers and compared with standard suspension cultures. In this chapter it is shown that the two different Cytopore microcarrier species can induce different cell growth patterns and volumetric production of two human recombinant glycoproteins (interferon-beta and tissue plasminogen activator).

4.2. Results:

4.2.1. Cytopore 1: Cell growth in Cytopore 1 Spinner flasks

The growth characteristics of two independent CHO cell lines transfected with the recombinant human IFN-beta gene and the human tPA gene were compared in stirred suspension cultures and in cultures containing Cytopore 1 macroporous microcarriers at concentrations between 0.25 mg/mL and 2 mg/mL. Each culture was inoculated with 10^5 cells/mL in serum-free medium (100 mL) in spinner flasks with continuous stirring at 45 rpm. Figure 4.1 shows growth profiles for the beta-interferon and tPA producing cell lines grown in Cytopore 1 culture. The maximum cell yield for suspension IFN-beta producing cells was 5.02×10^6 cells/mL and tPA cells was 4.28×10^6 cells/mL which occurred in at the end of the culture (day 7). The highest yield of cells in microcarrier cultures was different for the two different cell lines, where IFN-beta cells had a maximum of 3.00×10^6 cells/mL in Cytopore concentrations between 0.5 to 2 mg/mL, while tPA cells had a maximum yield of 4.98×10^6 cells/mL in 2 mg/mL Cytopore 1. The tPA cells showed a minimum cell yield in 0.25 mg/mL Cytopore 1 cultures of 2.30×10^6 cells/mL, while the IFN-beta cells showed a minimum cell yield of 1.75×10^6 cells/mL

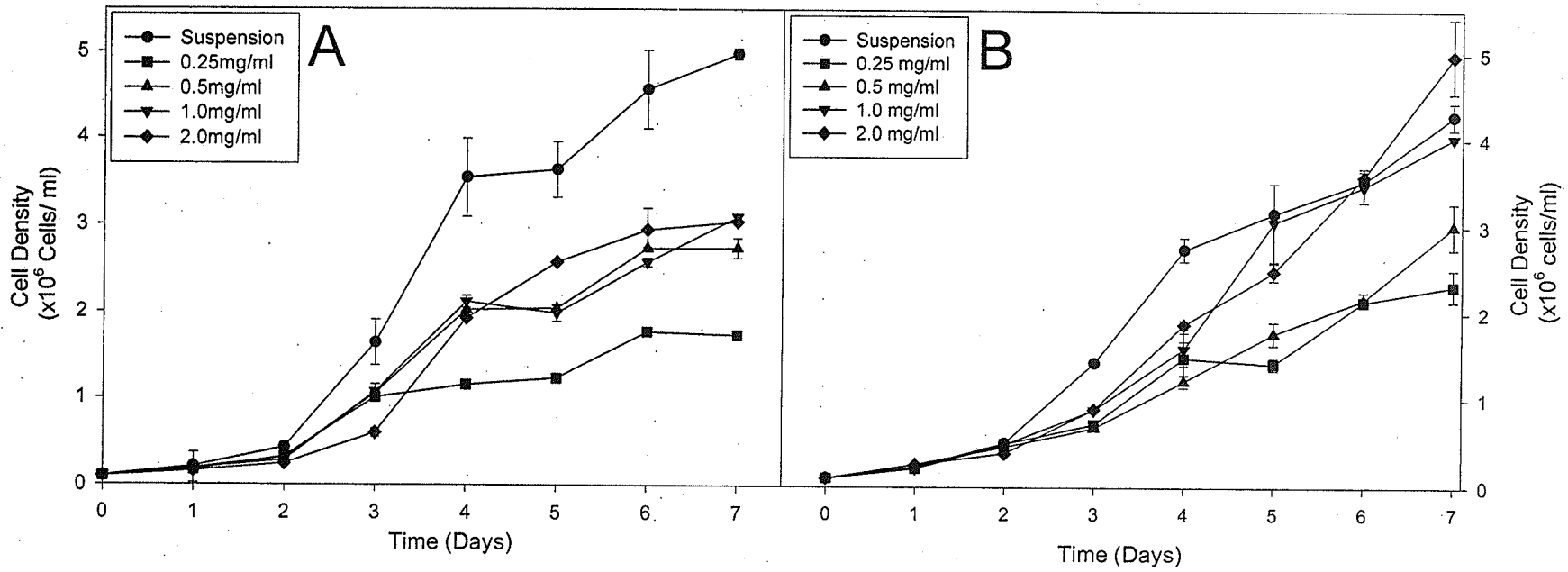

Figure 4.1: Growth profiles of IFN-beta (A) and tPA (B) producing CHO cells on Cytopore 1 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25 mg/mL (■), 0.5 mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 1 microcarriers or no microcarriers (●). The values are expressed as the cell densities in the culture where the samples are means ± SEM between duplicate cultures.

also in the lowest microcarrier cultures. The difference in cell yield between the different cell lines show that the microcarrier concentrations can be optimized between the cell lines for growth.

For the Cytopore cultures, cells became entrapped within the porous microcarriers within two hours of inoculation. Figure 4.2 shows the concentrations of cells that were found in suspension throughout the culture period for IFN-beta (A) and tPA (B) Cytopore 1 microcarrier cultures. In the lower concentrations of microcarriers, over time the IFN-beta cell numbers increased from no cells in suspension to $0.5x10^6$ cells/mL found in suspension. The other concentrations of Cytopore led to minimal cells in suspension (less than $0.2x10^6$ cells/mL). In the tPA cultures, $1.1x10^6$ cells/mL with 0.25 mg/mL microcarrier concentration were found to grow in suspension by the end of the culture. However, at other Cytopore 1 concentrations there were no other tPA cells found to grow in suspension throughout the culture period. During the first two days of culture the supernatants of the microcarrier cultures were visibly clear with no evidence of non-attached cells growing in suspension. At the peak cell density in cultures with the highest Cytopore concentration, the proportion of non-attached cells in culture was only 4.7 % as compared to 30-50 % for the culture at the lowest Cytopore concentration.

The mesh of cellulose present in the Cytopore allows for the entrapment of cells within the microcarrier. The cell density within the culture can be manipulated to allow for the estimation of cells that would occupy a microcarrier. Figure 4.3 shows the concentration of cells determined to occupy Cytopore 1 microcarriers. The lowest concentration of microcarriers (0.25mg/mL) shows the maximum cell to bead occupancy

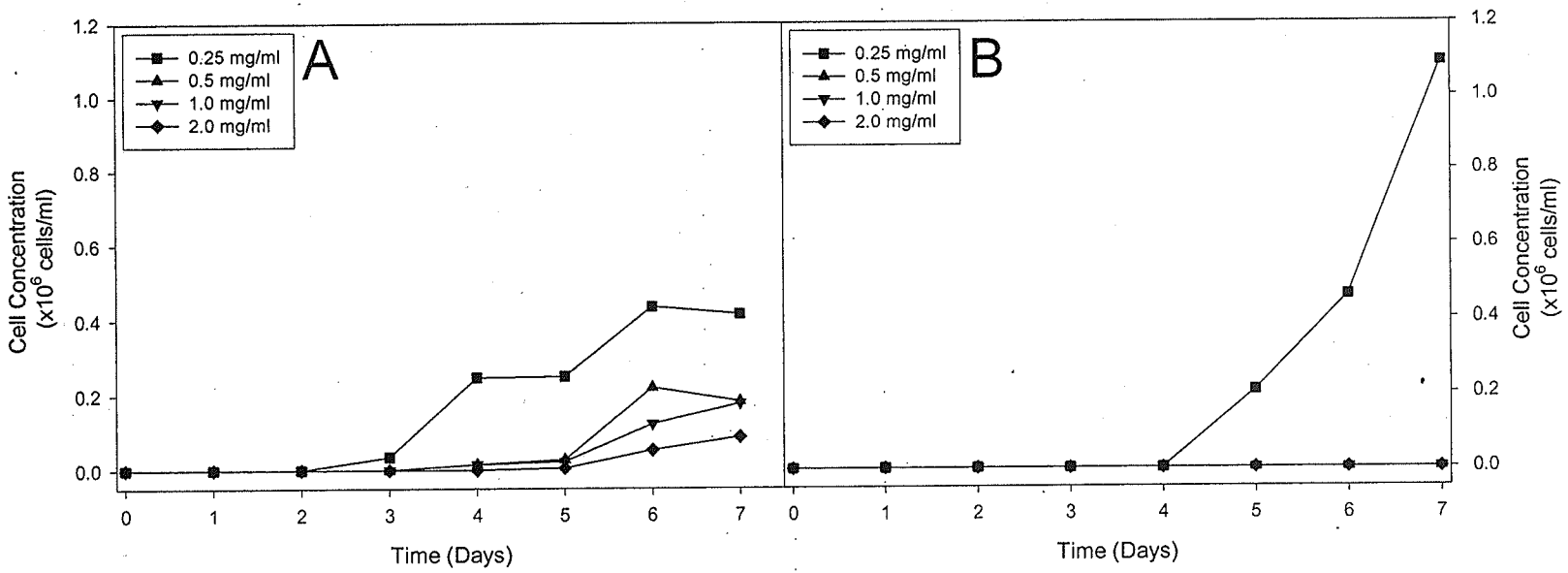

Figure 4.2: The cells found in suspension for Cytopore 1 cultures of cells producing IFN-beta (A) and tPA (B). The symbols represent cultures with varying concentrations of microcarriers 0.25 mg/mL (■), 0.5 mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 1 microcarriers. The values are expressed as means of duplicate samples.

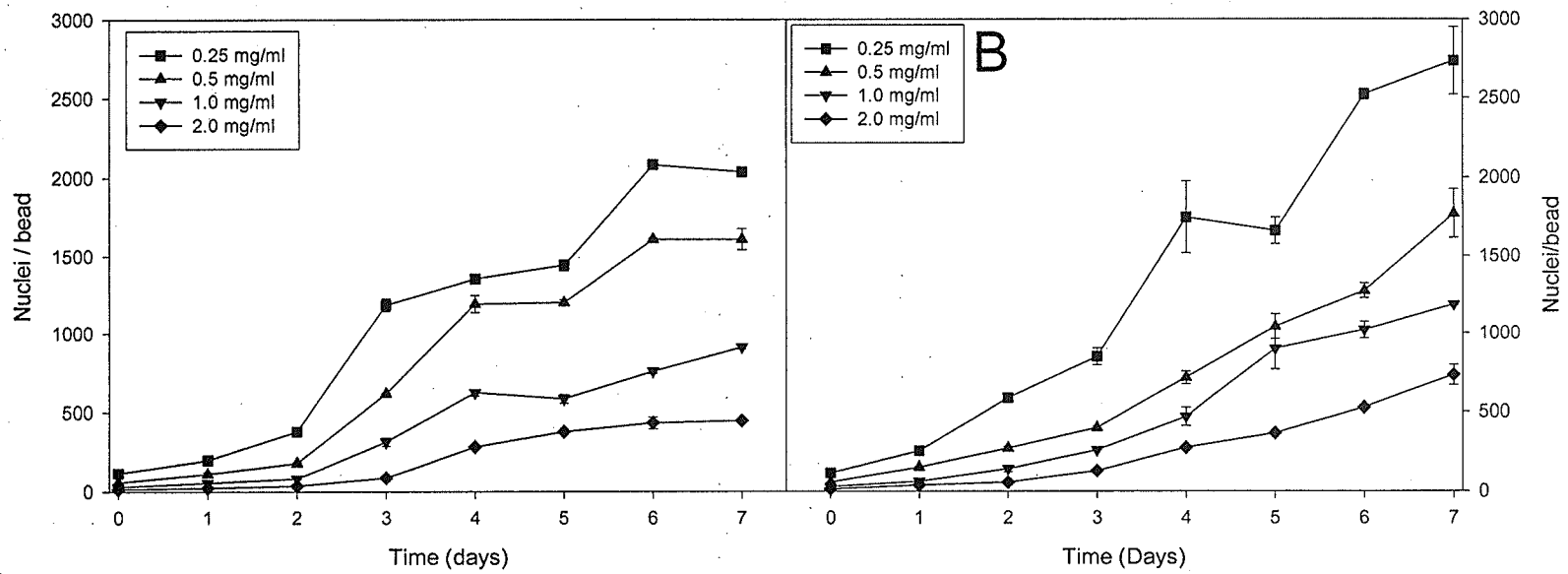

Figure 4.3: Nuclei to bead ratios for IFN-beta (A) and tPA (B) producing CHO cells on Cytopore 1 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25 mg/mL (■), 0.5 mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 1 microcarriers. The values are expressed as the cells attached to each microcarrier (calculated using values from Yokomizo *et al.*, 2004) where the samples are means ± SEM between duplicate cultures.

in each cell line, while the highest microcarrier concentration (2mg/mL) shows the minimal cell to bead occupancy. For the IFN-beta producing cell line the maximum cell to bead ratio is close to 2000 cells/bead, while the tPA producing cell line show over 35 % greater occupancy with 2750 cells/bead.

4.2.2. Cytopore 1: IFN-beta and tPA Production

In the case of IFN-beta production, ELISA determinations were made before and after denaturation of culture samples. Figure 4.4 shows the production of native (A) and denatured (B) IFN-beta from microcarrier and suspension cultures. As mentioned in Appendix A, due to the hydrophobicity of the IFN-beta protein, the protein has the tendency to aggregate, thus it is necessary to determine the titers of IFN-beta from native protein samples and from denatured protein samples. This calculation allows for the determination of aggregation which occurs within the culture. The suspension IFN-beta culture gives a maximum yield of 2.4×10^6 units/mL native IFN-beta, and a corresponding value of 4.04×10^6 units/mL denatured protein.

It is desirable to design a culture process that eliminates product aggregation, and thus the different systems for increasing protein production must be monitored for aggregation. Figure 4.5 shows the percent product aggregation at day 6 of the IFN-beta titers in each culture. The aggregation of IFN-beta in suspension cultures at day 6 is 33.6 %, compared to Cytopore 1 cultures with aggregation ranging from 5.1 to 22.2 %. The aggregation for Cytopore cultures appear to increase with the concentration of microcarriers.

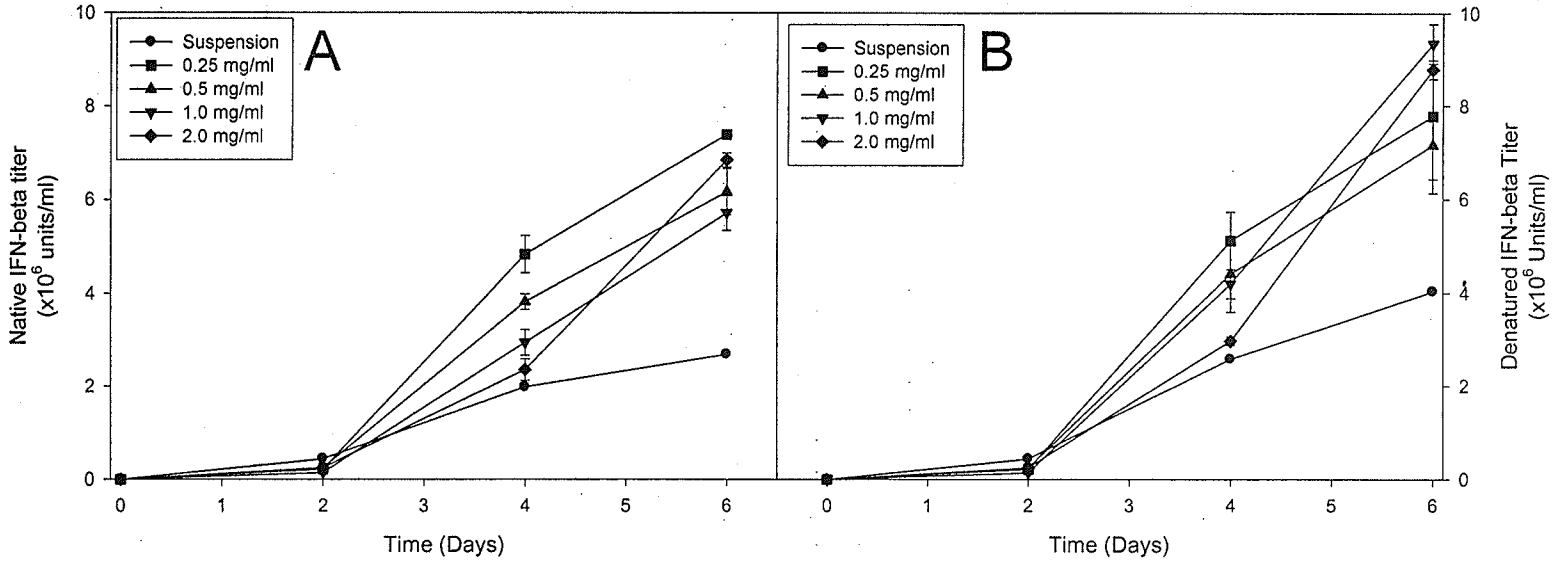

Figure 4.4: Native IFN-beta (A) and denatured IFN-beta titers from Cytopore 1 microcarrier and suspension cultures. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25mg/mL (■), 0.5mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 1 microcarriers or no microcarriers (●). The titers were as calculated from ELISAs where the values are means ± SEM between duplicate cultures.

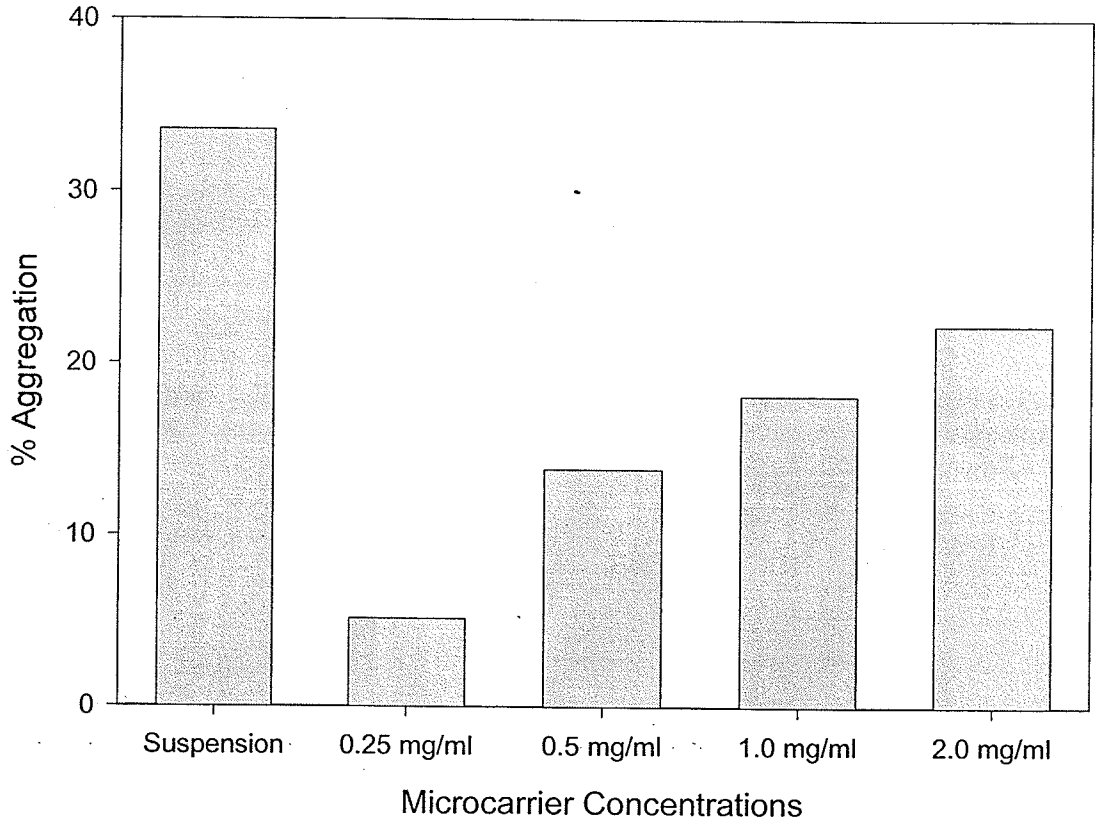

Figure 4.5: Aggregation at day 6 of Cytopore 1 microcarrier culture. The aggregation is quantified as a comparison of native and denatured protein quantities as estimated by the ELISA method. The values are expressed as the aggregation of duplicate samples ± SEM.

The production of tPA quantified by ELISA is shown in Figure 4.6. The protein yield for suspension cultures was 3200 Units/mL at day 7 of the culture. However, the secreted protein appeared to increase significantly from the beginning of the culture to day 5, after which the protein increases by less than 10 % for the duration of the culture to day 7. The lowest concentration of Cytopore 1 (0.25mg/mL) gave a maximum titre of 5255 Units/mL at day 5, but slowly declined to 3656 Units/mL by day 7. This decrease is believed to be due to a combination of tPA degradation as well as reduced synthesis (as explained in Appendix A). The other concentrations of Cytopore 1 gave tPA titers between 5250 to 6790 Units/mL at day 7 of the culture, with 1.0 mg/mL of Cytopore leading to the maximum titre.

Protein titers were normalized against the suspension culture to allow for comparisons between samples as well as different proteins, where the suspension culture was given a value of 1 unit/mL. The normalization gives us the relative titre increase (Figure 4.7).

4.2.3. Cytopore 2: Cell growth in Cytopore 2 Spinner flasks

The growth of cells in Cytopore 2 microcarriers was also investigated. The main difference between Cytopore 1 and 2 is the difference in the charge of the bead. While the charge density of Cytopore 1 microcarriers is 1.1 meq/g, the charge density of Cytopore 2 is slightly higher at 1.8 meq/g. This higher charge density suggests that cells would have a higher affinity for the Cytopore 2 microcarrier due to the net negative charge of the membrane.

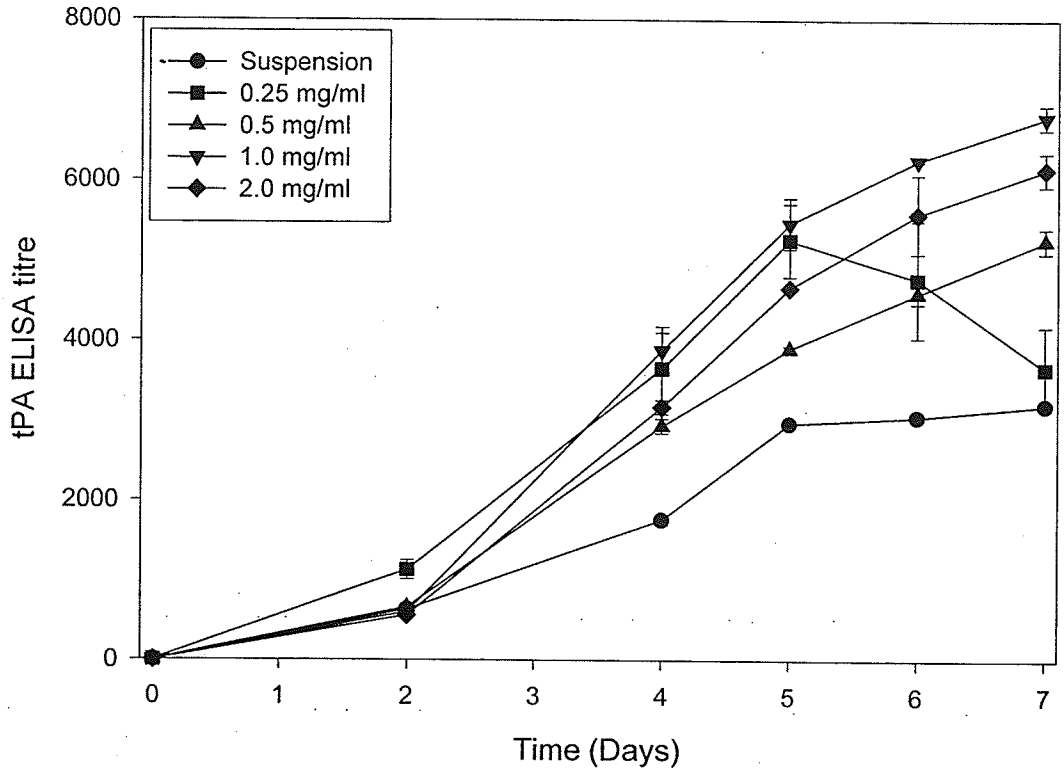

Figure 4.6: tPA produced in CHO cells on Cytopore 1 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25mg/mL (■), 0.5mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 1 microcarriers or no microcarriers (●). Protein was quantified using ELISAs where the samples are means ± SEM between duplicate cultures.

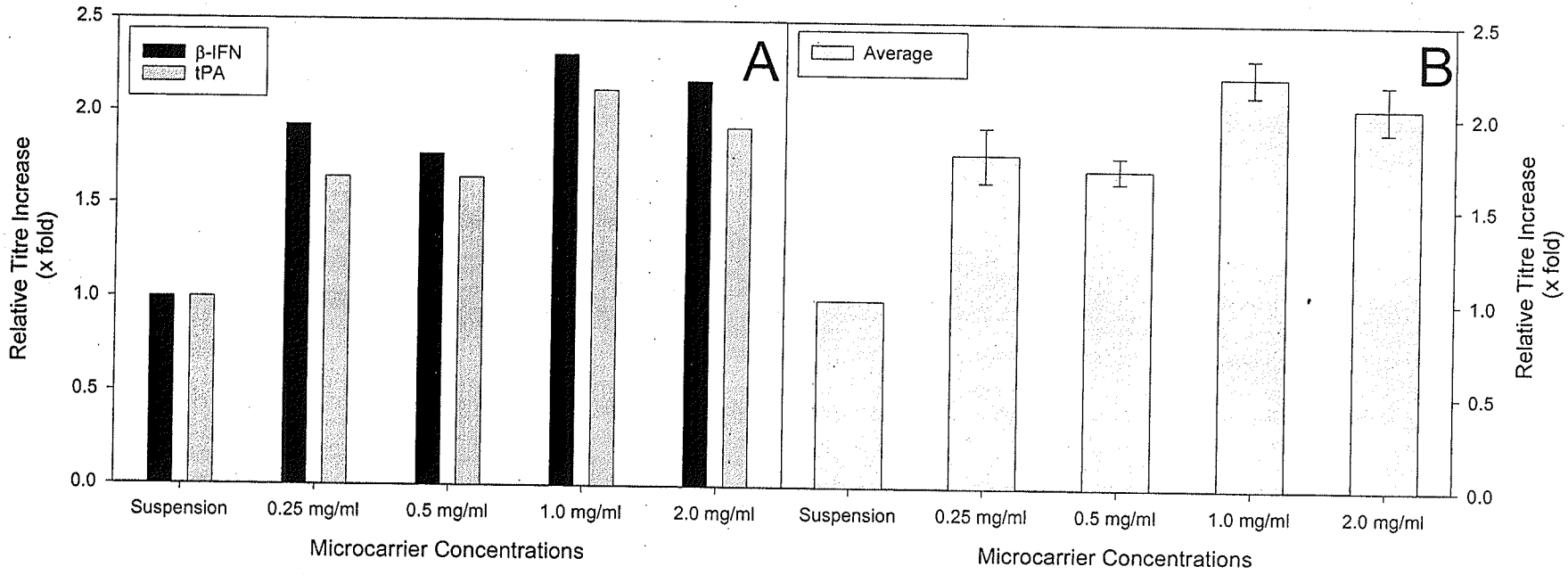

Figure 4.7: Relative maximum protein titers from Cytopore 1 cultures, normalized to the suspension culture titre. The titers were normalized as compared to the suspension protein yield. Panel A represents the overall fold increase for the production of IFN-beta and tPA and panel B shows the average fold increase of both the cell lines ± SEM.

The growth of IFN-beta producing cells (Figure 4.8, panel A) in suspension followed a similar growth pattern as the suspension culture in the Cytopore 1 data set. Where the cell density increased for the first 6 days, with a maximum cell yield of $4.15x10^6$ cells/mL then a decline of 10 % to $3.75x10^6$ cells/mL over the last day of the culture. The microcarrier cultures all followed a similar growth pattern to the Cytopore 1 cultures (Figure 4.1, panel A) where the 0.5 mg/mL and 1.0 mg/mL yielded a slightly higher cell yield at $3.50x10^6$ and $3.28x10^6$ cells/mL, respectively. At 2.0 mg/mL microcarriers the cell concentration at day 7 was $2.40x10^6$ cells/mL, while the 0.25mg/mL microcarrier culture yielded a cell concentration of $1.2x10^6$ cells/mL. The growth of the tPA cells showed a similar growth pattern, as shown in Figure 4.8B. The cell density for the microcarrier cultures ranged from $2.99x10^6$ cells/mL to $3.81x10^6$ cells/mL, while the suspension culture had a maximum of $4.28x10^6$ cells/mL at day 7. Similarly the lowest concentration induced a lower growth, reaching a maximum of $1.8x10^6$ cells/mL at day 7.

Figure 4.9 indicates the cells found to grow in suspension at the varying concentrations of microcarriers where Panel A shows IFN-beta producing cells, and Panel B, the tPA producing cells. In both cases, at 0.25mg/mL Cytopore 2 the cell concentration in suspension was over $1x10^6$ cells/mL at day 7 of the culture. The IFN-beta producing cells showed a slight increase of cells in suspension beginning at day 4. However the growth of cells in suspension was not significant compared to the overall cell concentration. The maximum cell concentration in suspension of the other concentrations of Cytopore 2 was less than $0.2x10^6$ cells/mL compared to $3.0x10^6$ to $3.5x10^6$ cells/mL total. tPA producing cells had a higher quantity of cell in suspension compared to the IFN-beta producing cells. The maximum cell yield in suspension was

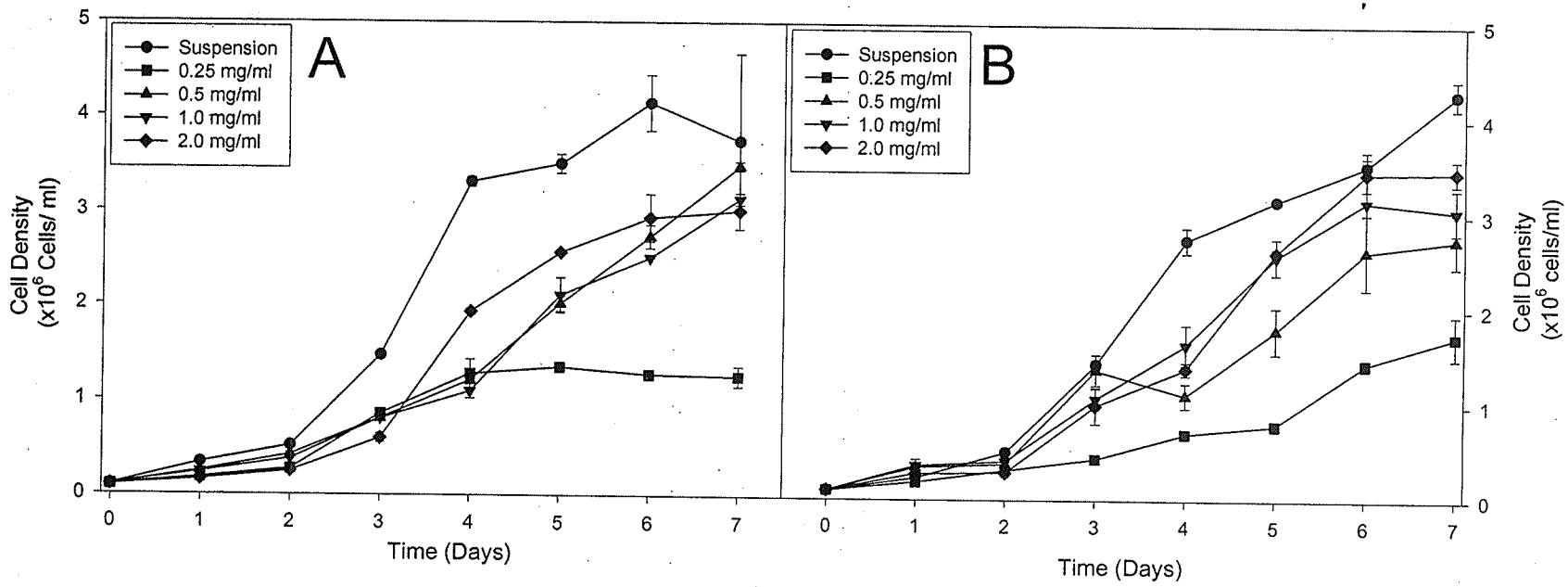

Figure 4.8: Growth profiles of IFN-beta (A) and tPA (B) producing CHO cells on Cytopore 2 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25 mg/mL (■), 0.5 mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 2 microcarriers or no microcarriers (●). The values are expressed as the cell densities in the culture where the samples are means ± SEM between duplicate cultures.

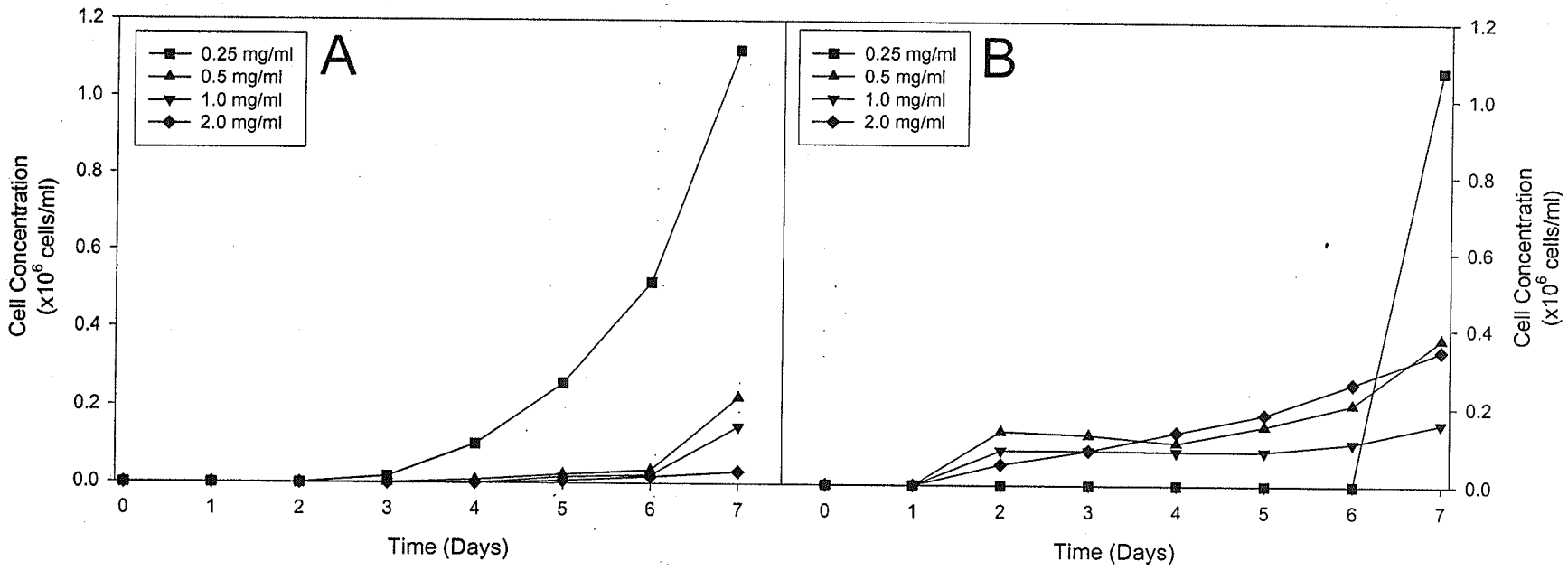

Figure 4.9: The cells found in suspension for Cytopore 2 microcarrier cultures of cells producing IFN-beta (A) and tPA (B). The values are expressed as means of duplicate samples.

$0.4x10^6$ cells/mL in the 0.5mg/mL and 2.0 mg/mL Cytopore 2 concentrations. The cells in suspension at each Cytopore 2 microcarrier concentration was about 10 % except the lowest microcarrier concentration (0.25mg/mL) where 35-50 % of cells were found in suspension by day 7.

A comparison of the cells to microcarriers (Figure 4.10) show an increase in the cell to bead occupancy in the 0.25mg/mL concentration in the case of the IFN-beta cells. This increase occurs until day 4, after which point the cell to bead ratio remains near 1700 cells/beads for the duration of the culture. However, in the 0.5mg/mL IFN-beta culture, the ratio increases to 2000 cells/bead. In the case of the tPA cell line the maximum cell to bead ratio is 2000 cells/bead, thus suggesting that there is a maximum of 2000 cells per bead. In the majority of cases, the beads are not fully occupied.

4.2.4. Cytopore 2: IFN-beta and tPA Production

IFN-beta was measured as native and denatured protein. In the panel A of Figure 4.11, there is a maximum native protein yield of $7.5x10^6$ units/mL by the cells in 0.5mg/mL Cytopore 2 concentration, which is just over 2.8-fold greater than the suspension protein yield. The other Cytopore concentrations induce a 1.4 to 2.1-fold higher protein yield than the suspension culture. Panel B shows the denatured protein yield from the suspension and Cytopore cultures. The denatured protein titre of the Cytopore cultures was 1.2 to 2.5-fold greater than the yield of the suspension cultures with $4.04x10^6$ units/mL.

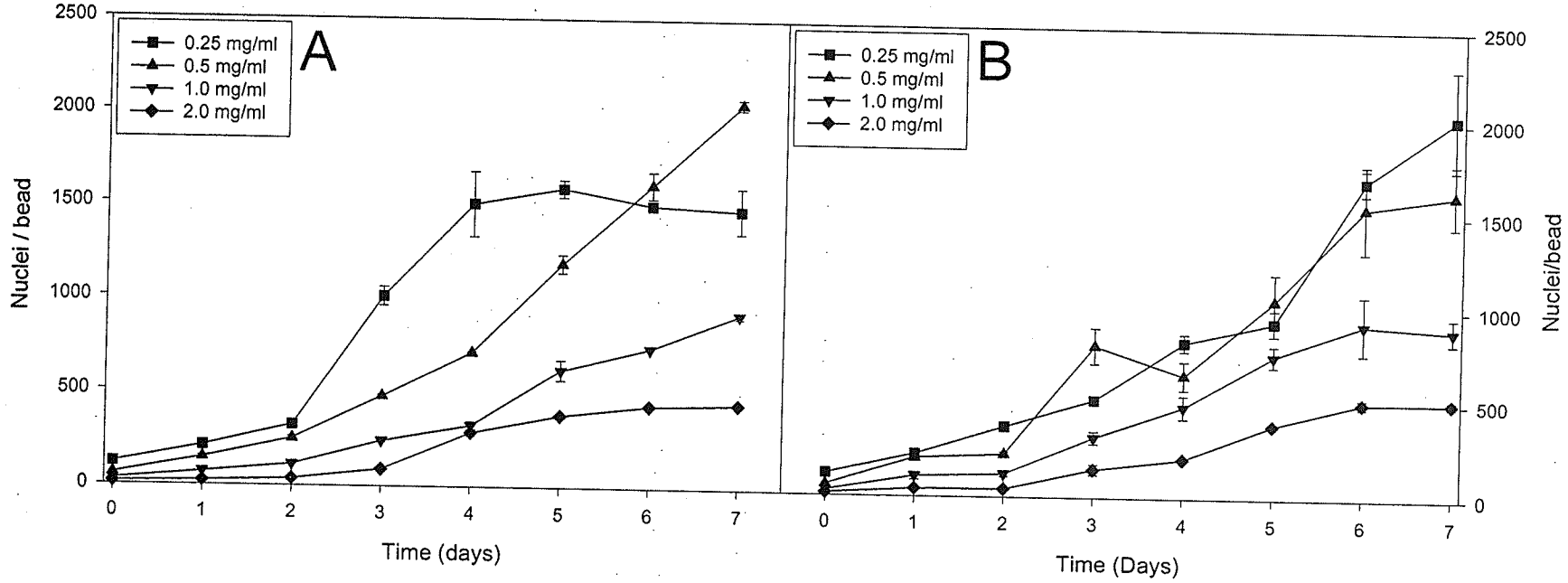

Figure 4.10: Nuclei to bead rations for IFN-beta (A) and tPA (B) producing CHO cells on Cytopore 2 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25mg/mL (■), 0.5mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 2 microcarriers. The values are expressed as the cells attached to each microcarrier (calculated using values from Yokomizo *et al.*, 2004).

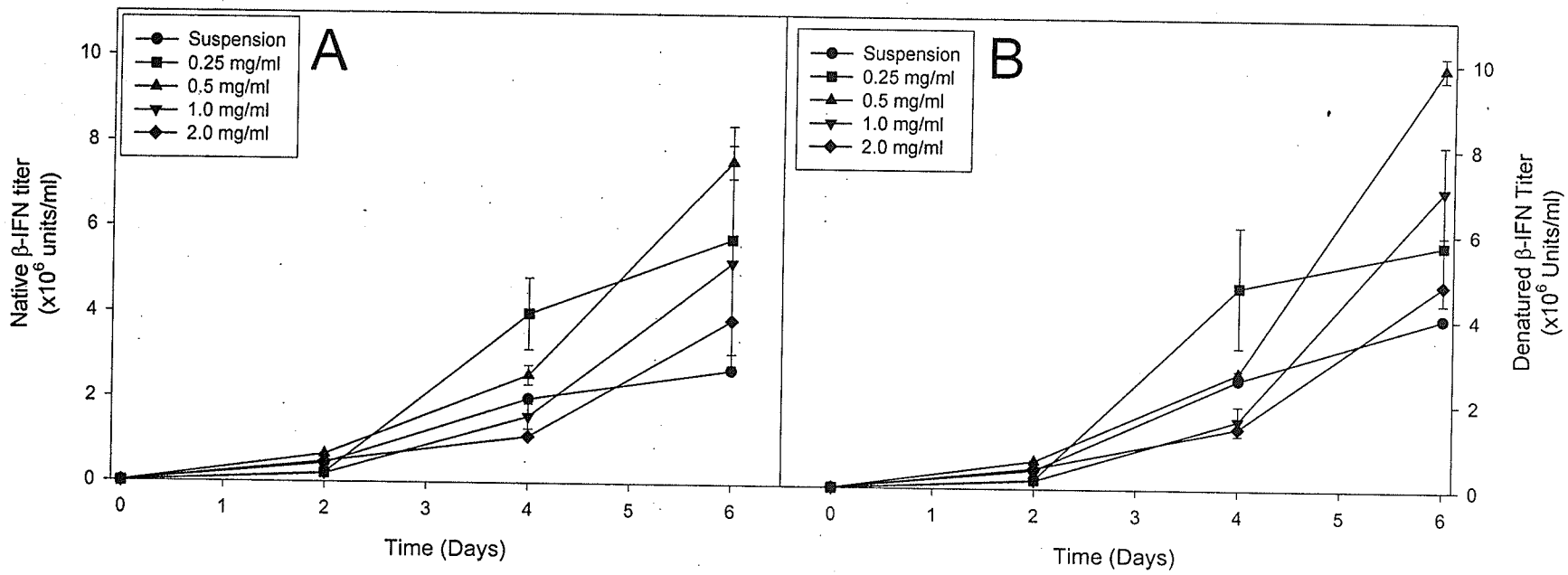

Figure 4.11: Native IFN-beta (A) and denatured IFN-beta titers from Cytopore 2 microcarrier and suspension cultures. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25mg/mL (■), 0.5mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) Cytopore 2 microcarriers or no microcarriers (●). The titers were as calculated from ELISAs where the values are expressed as means of duplicate samples ± SEM.

Comparison of aggregation of IFN-beta (Figure 4.12) shows that 33 % of the suspension culture protein was aggregated by day 6 of the culture. Although the aggregation of the IFN-beta in the microcarrier cultures did not reach the levels of the suspension culture; the aggregation was between 20 to 25 % in all cases, except 0.25 mg/mL Cytopore. The aggregation of IFN-beta in the 0.25 mg/mL at day 4 was 16 %. However, in the last 2 days of the culture the level of aggregated protein produced must have been negligible compared to the overall protein, as the titers from the ELISA response showed no significant protein aggregation at day 6.

The maximum protein yield of tPA (Figure 4.13) was obtained in the 0.5mg/mL Cytopore 2 culture. Where the 0.25 mg/mL and 1.0 mg/mL showed a maximum protein yield at day 6 of the culture, with over 6000 Units/mL, the yield dropped by over 30 % to about 4000 Units/mL by day 7. Thus the 0.5 mg/mL concentration of Cytopore 2 appears to give a higher product yield compared to the suspension culture, yielding over 2-fold greater tPA.

The information from Figures 4.11 and 4.13 can be summarized in Figure 4.14. Where the left panel (A) shows the relative titre increase of the IFN-beta and tPA produced in Cytopore cultures, standardized to the suspension cultures. Panel B shows the average product yield between the standardized proteins. It would appear that the optimal concentration of Cytopore 2, for a maximum protein yield is 0.5mg/mL, compared to 1.0 mg/mL for Cytopore 1 microcarriers.

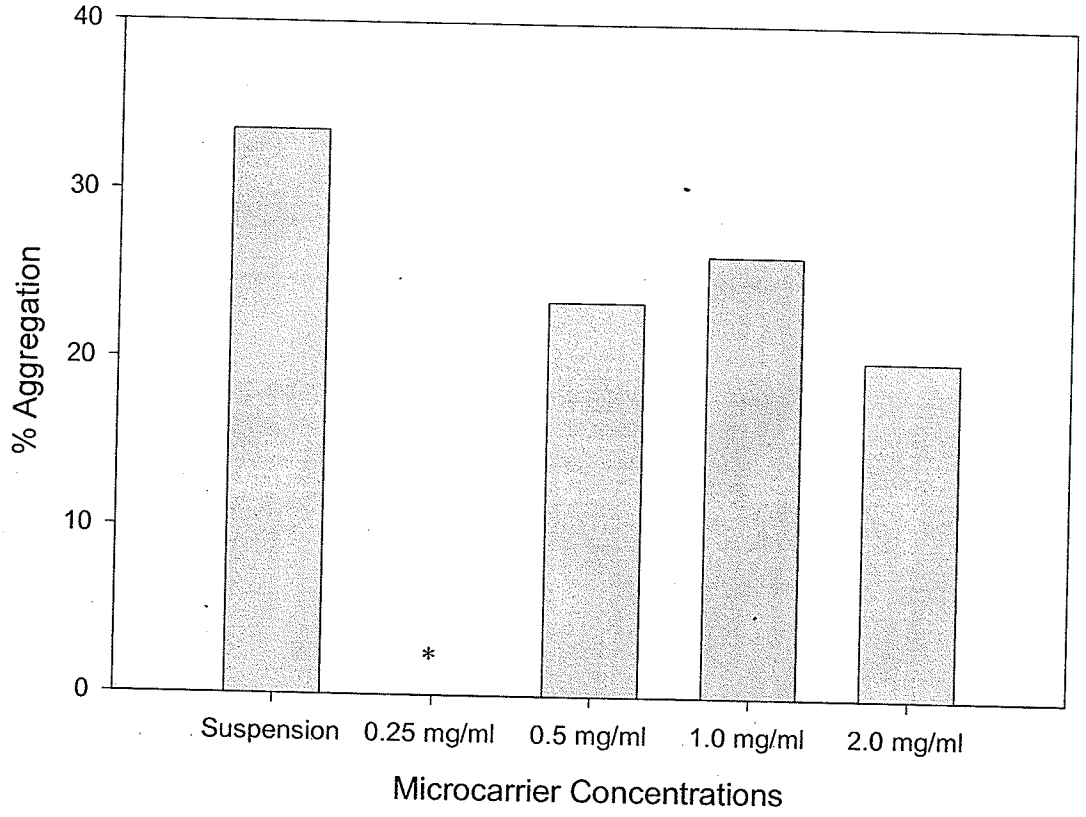

Figure 4.12: Aggregation of IFN-beta in Cytopore 2 at day 6 of culture. The aggregation is quantified as a comparison of native and denatured protein quantities as estimated by the ELISA method. The values are expressed as means of duplicate samples.* represents negligible aggregation.

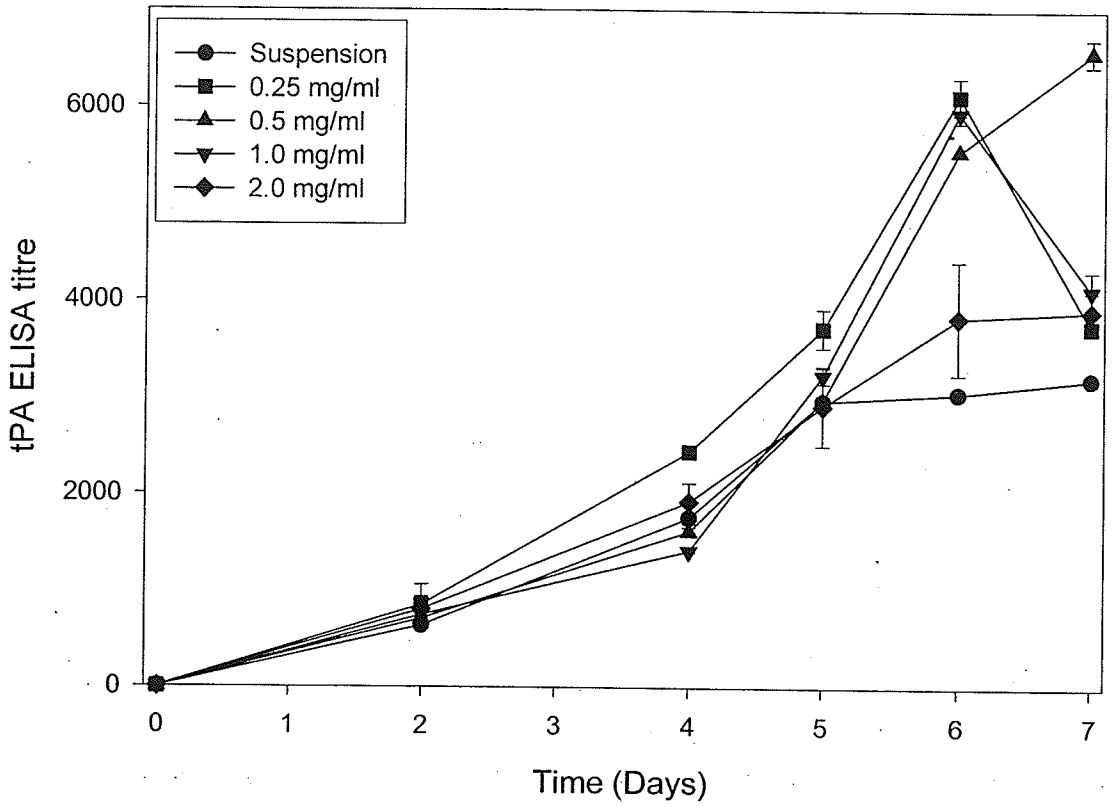

Figure 4.13: tPA produced in CHO cells on Cytopore 2 microcarriers. Cells were inoculated at $1x10^5$ cells/mL into culture medium (100 mL) containing 0.25 mg/mL (■), 0.5 mg/mL (▲), 1.0 mg/mL (▼) and 2.0 mg/mL (◆) microcarriers or no microcarriers (●). The titers were as calculated from ELISAs where the values are expressed as means of duplicate samples ± SEM.

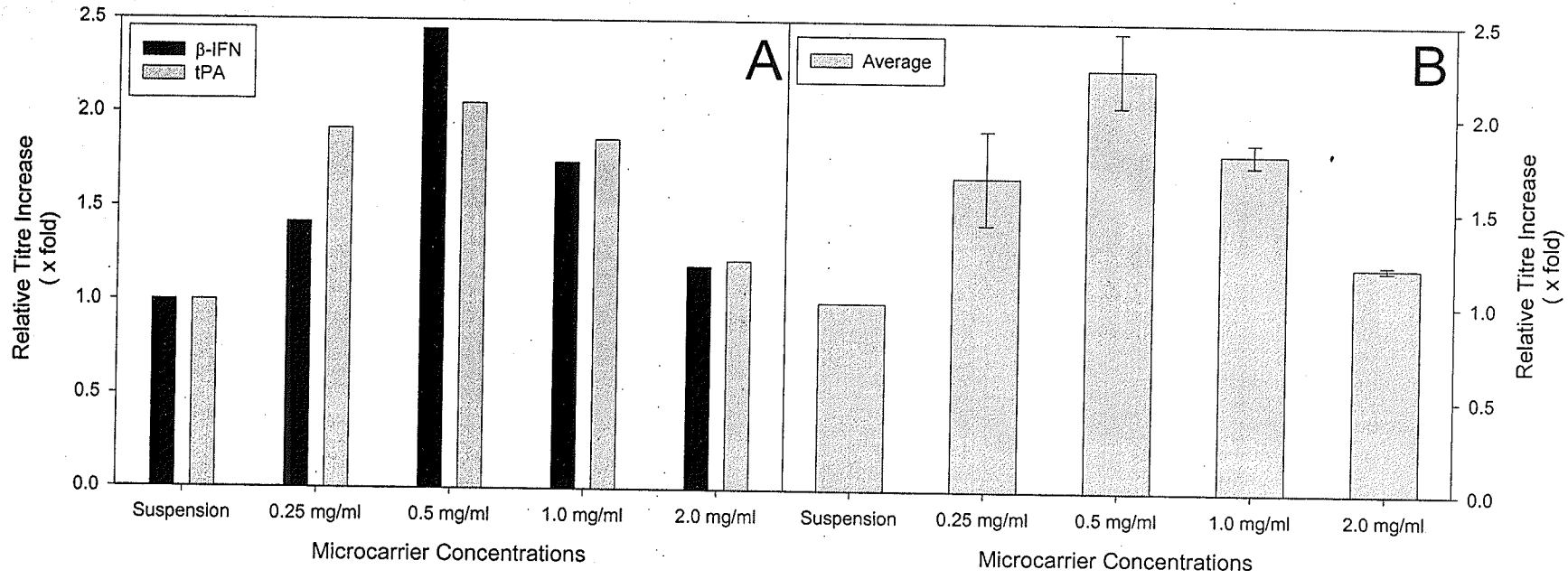

Figure 4.14: Relative maximum protein titers from Cytopore 2 cultures as compared to the suspension culture titre. The titers were normalized as compared to the suspension protein yield. Panel A represents the overall fold increase for the production of IFN-beta and tPA and panel B shows the average fold increase of both the cell lines ± SEM.

4.2.5. Comparing Cytopore 1 and Cytopore 2

The productivity of IFN-beta and tPA from CHO cells in Cytopore 1 and 2 microcarriers are summarized in Table 4.1. The protein titers were normalized against the suspension protein titre for each protein (IFN-beta and tPA), where the suspension protein titre was termed 1 unit/mL. The protein titers increased with the concentration of microcarriers, where the optimal concentration of Cytopore 1 was 1.0 mg/mL for the production of both IFN-beta and tPA, and the optimal concentration of Cytopore 2 was 0.5mg/mL for the production of both recombinant proteins.

4.3. Discussion

These titers of native and denatured protein were up to 50 % less than the titers calculated for Cytopore microcarrier cultures, which range between 5.71×10^6 to 7.38×10^6 units/mL native protein and 7.15×10^6 to 9.35×10^6 units/mL denatured protein.

Many studies have reported the benefits of cell growth in Cytopore microcarriers. The use of Cytopore microcarriers has been especially beneficial for perfusion bioreactor cultures where media is exchanged while cells remain within the culture. The use of Cytopore has increased the efficiency of these systems for the production of recombinant proteins, easing the cell retrieval from cultures. Zhaolie *et al.* (1999) have reported high CHO cell densities of 8.83×10^6 cells/mL attained after 20 days. The cultures appeared to be healthy at the end of the 20 day period with multiple layers forming on the microcarrier and cell clumping within the carrier. Common CHO cell densities attained in Cytopore cultures reach over 1×10^7 cells/mL as reported by Hu *et al.* (2000) and Chen *et al.* (2000), and as high as 2×10^7 cells/mL reported by Cong *et al.* (2001). While our

Table 4.1: Summary table of the productivity of IFN-beta and tPA producing CHO cell lines in Cytopore 1 and 2 microcarrier cultures in 100mL spinner flasks. Protein was quantified using an ELISA. IFN-BETA samples were quantified by native protein yield (non-denatured) and denatured (total protein). tPA samples were quantified by ELISA of supernatant samples.

Cytopore	Microcarrier Conc.(mg/mL)	Native IFN-beta titre (Normalized Units/mL)	Denatured IFN-beta tire (Normalized Units/mL)
1	Susp	1.00	1.51
1	0.25	2.75	2.90
1	0.5	2.30	2.67
1	1.0	2.85	3.49
1	2.0	2.55	3.28
2	Susp	1.00	1.51
2	0.25	2.14	2.14
2	0.5	2.83	3.69
2	1.0	1.94	2.63
2	2.0	1.44	1.80
Cytopore	Microcarrier Conc.(mg/mL)	tPA titre (Normalized Units/mL)	
1	Susp	1.00	
1	0.25	1.64	
1	0.5	1.64	
1	1.0	2.12	
1	2.0	1.92	
2	Susp	1.00	
2	0.25	1.92	
2	0.5	2.05	
2	1.0	1.86	
2	2.0	1.22	

cultures were batch cultures, and thus stopped at day 7, we were able to obtain densities as high as $5x10^6$ cells/mL bead. Feeding the cultures either by inducing a fed-batch system, or perfusion culture may allow for a higher cell yield close to those obtained by Cong *et al.* (2001) or Chen *et al.* (2000).

Several other macroporous carriers have been used to immobilize and grow mammalian cells. As an example, Cultispher G is a degradable crosslinked gelatin based macroporous microcarrier that has been used for the production of recombinant proteins and viruses (Mignot *et al.*, 1990; Berry *et al.*, 1999). This has been reported to accommodate high cell/ bead ratios (>4,000) and high cells densities (> 10^8 cells/mL) in stirred tank bioreactors. Chen *et al.*, (2003) showed that the growth of osteocarcinoma cells in a fibrous matrix to high densities ($3x10^8$ cells/mL) led to selective retention of healthy non-apoptotic cells, mostly arrested in the G1/ G0 phase. The cell densities reached in this study was similar to that reported previously. A comparison of the cells to the microcarriers, give maximum ratios ranging between 2000 to 2750 cells/bead.

The increase in cell growth has been shown to benefit the production of recombinant proteins from these immobilized cultures. Zhaolie *et al.* (1999) reported a maximum product yield of 12473 Units/mL of tPA reached at the end of a 20 day culture. Similarly Hu *et al.* (2000) reported a maximum product yield of 7335 Units/mL of urokinase type-plasminogen activator from a Cytopore bioreactor culture after 26 days. The phenomenon of enhanced productivity from cells grown to high densities in a microenvironment has been previously observed for monoclonal antibodies secreted by murine hybridomas (Lee *et al.*, 1991) and from transfected BHK cells (Yamaguchi *et al.*, 1997).

The difference in the growth of the IFN-beta producing CHO cell line and the tPA producing cell line can be explained by cell line dependence. Although we had a similar protein yield with varying concentrations of Cytopore microcarriers, others have reported differently. Nam *et al.* (2007a) reported that there was no enhancement in accumulated recombinant protein activity in Cytopore 1 cultures in which tPA showed a similar rate of production as in suspension and SEAP produced in suspension was higher than 53 % greater than the Cytopore 1 cultures. Mignot *et al.* (1990) showed that there was no enhancement in production of Von Willebrand Factor from CHO cells grown in Cultispher G cultures.

The use of Cytopore for the maximization of recombinant IFN-beta and tPA from CHO cell culture was found to be beneficial. We report an increase of up to 2.5-fold of recombinant protein yields in both cases of protein production. As well, in the case of IFN-beta, protein aggregation was decreased in the presence of Cytopore compared to the aggregation in the suspension cultures. Therefore we decided to use Cytopore in further studies to increase production of proteins in bioreactor cultures.

Chapter 5

5. Relationship of cell density and specific productivity within microcarriers*

5.1. Introduction

In order to develop bioprocesses suitable for the large-scale production of proteins it is essential to be able to isolate genetically-engineered producer cells with high specific productivity and a capability for their growth to high densities in culture (Butler 2005; Wurm 2004). In particular the ability to maintain high densities of cells in a viable state for prolonged periods in culture has been a key factor in the increased volumetric productivities that have been observed in such bioprocesses over the last 20 years (Wurm 2004). These higher productivities have to some extent off-set the predicted short-fall in volumetric bioreactor capacity world-wide (Butler 2005; Mallik *et al.* 2002). Thus, improvements in the efficiency of cultures allows the production of high quantities of protein and reduces the requirements for volumetric scale-up of bioreactors.

Macroporous microcarriers were designed to offer internal spaces where cells may be anchored or embedded and are able to grow to high cell densities in a pseudo-suspension culture. The entrapped cells within the carriers can be protected from high shear forces. The macroporous microcarriers can allow higher cell to bead loadings which allow the microcarrier to reach high cell densities without being compromised by high spinner rotation speeds (Ng *et al.* 1996).

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* The content of this chapter was included in a paper: Tharmalingam, T., Sunley, K., Spearman, M., and M. Butler. **Enhanced production of human recombinant proteins from CHO Cells grown to high densities in macroporous microcarriers** (submitted to Biotechnology Progress, May 2008) Work reported in the paper completed by others were omitted from this chapter.

In this chapter, I investigate the relationship between an increase in cell density and the impact on the specific productivity of recombinant protein being produced within both CHO cell lines (producing IFN-beta and tPA).

5.2. Results

5.2.1. Cell density reached within microcarriers

The mesh of cellulose present in the Cytopore allows for the entrapment of cells within the microcarrier. This is evident in the micrographs shown in Figure 5.1, where the left panel shows a Cytopore bead at day 2 from a 1 mg/mL Cytopore culture. At this stage, the average microcarrier entraps approximately 50 cells (about $14x10^6$ cells/mL bead volume). As the culture progresses, the microcarrier becomes highly populated with the value increasing to approximately 400 cells/ bead (about $55x10^6$ cells/mL) at day 4.

The cell concentrations within the microcarriers are shown in Figure 5.2. The cell density within the microcarrier is the estimated density of the cells confined within the boundaries of the microcarrier; where the volume of the microcarrier was calculated to be $7.24x10^{-6}$ cm^3 and 95 % of the microcarrier is porous, resulting in an available volume of $6.52x10^{-6}$ cm^3 (refer to Materials and Methods, Section 2.7.1).

The cell density within the microcarrier at the beginning of the culture was calculated to vary between $2.26x10^6$ cells/cm^3 to $18.04x10^6$ cells/cm^3 as the overall inoculum cell concentration was kept constant in regards to the total culture volume. The cell concentration quoted above refers to the concentration of cells within the bead, compared to the inoculation cell density which was $1x10^5$ cells/mL culture volume. However, the

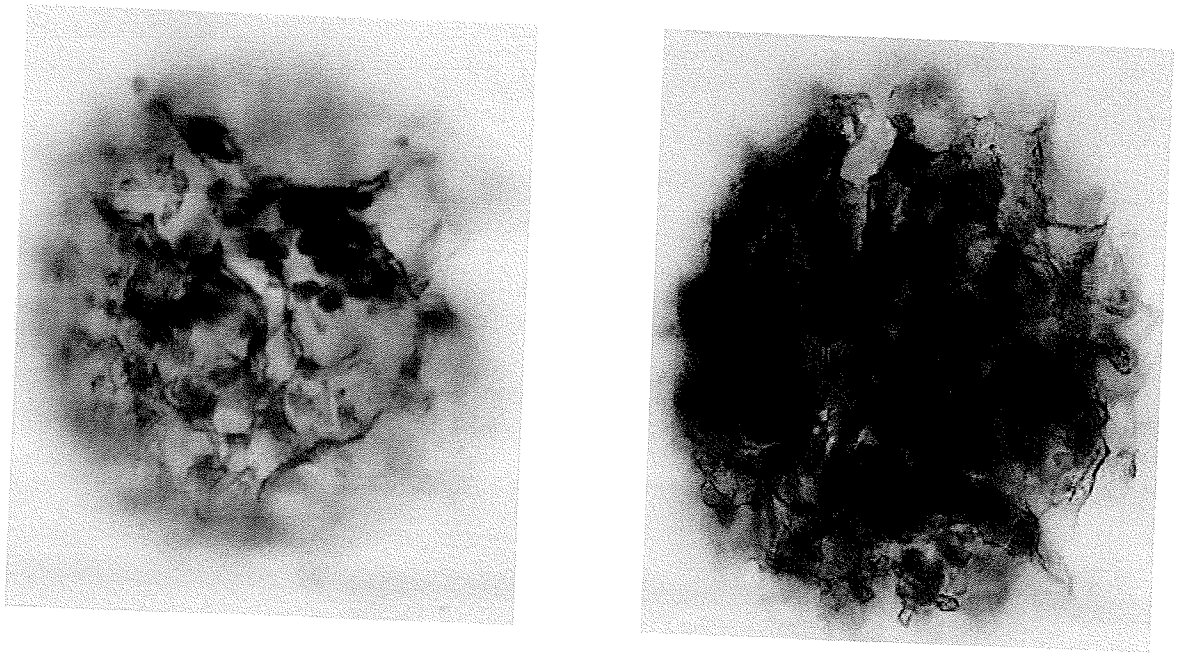

Figure 5.1: Brightfield photo micrograph of CHO cells grown in 1.0 mg/mL Cytopore 1. Left micrograph indicates a microcarrier with approximately 50 cells (day 2) and the right represents a microcarrier with approximately 400 cells (day 4). Microcarriers were stained with crystal violet.

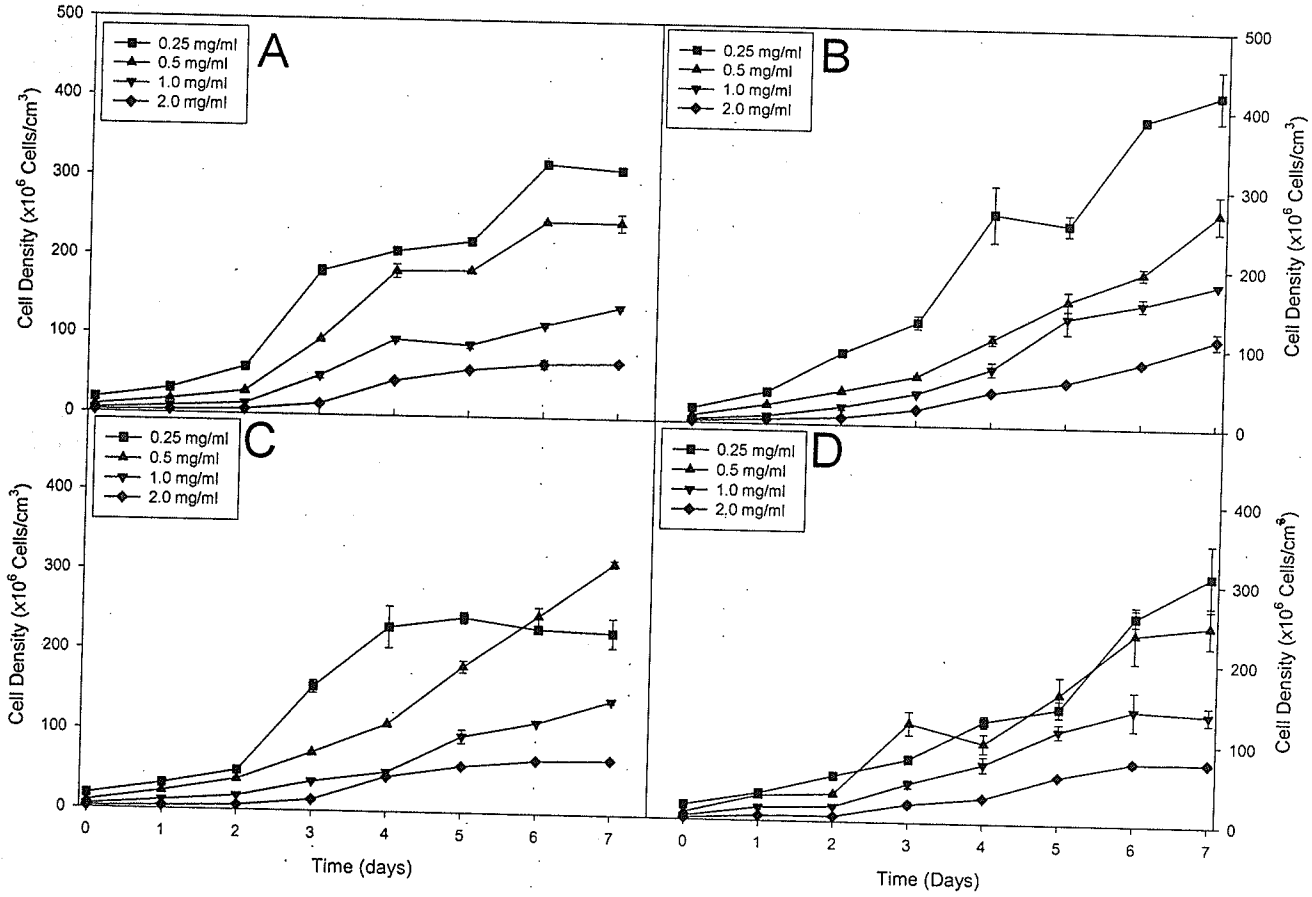

Figure 5.2: Cell density attained within Cytopore microcarriers for IFN-beta (A and C) and tPA (B and D) producing cell lines in varying concentrations of microcarriers (0.25mg/mL ■; 0.5mg/mL ▲; 1.0 mg/mL ▼;2.0 mg/mL ♦) and suspension cultures (●). Panels A and B show growth in Cytopore 1, whereas Panels C and D show growth in Cytopore 2 microcarriers. Values represent the means of 2 independent samples.

cells within the lowest concentration of Cytopore microcarriers (0.25 mg/mL) increased to a maximum of $400x10^6$ cells per milliliter of bead volume over a 7 day culture. In comparison, a suspension culture where maximum cell density reached at day 7 had approximately $5x10^6$ cells per milliliter of culture medium. This high cell density within the beads of the microcarrier cultures was evident with both CHO cells lines and for both microcarrier types.

5.2.2. Specific Productivity

The relationship between the cell density within the microcarriers and the cell-specific productivity of protein was determined from a plot of the specific productivity during the growth phase and the cell concentration within the microcarrier at day 4. Data from the production of both recombinant proteins were combined. The specific productivities were normalized against standard values set to 1 obtained in suspension cultures of each cell line. This allowed Q_P data from the two cell lines to be compared. The relationship between Q_P and cell density was determined by the best-fit plot using a polynomial algorithm (SigmaPlot). Acceptable profiles were established by maximization of r^2 values, where an r^2 value of 1 indicates a perfect fit.

The results are shown in Figures 5.3 and 5.4. For Cytopore 1 cultures (Figure 5.3) the pattern appeared to be hyperbolic following equation 1 below.

$y = 2.385x/(3.942 + x)$ equation 1

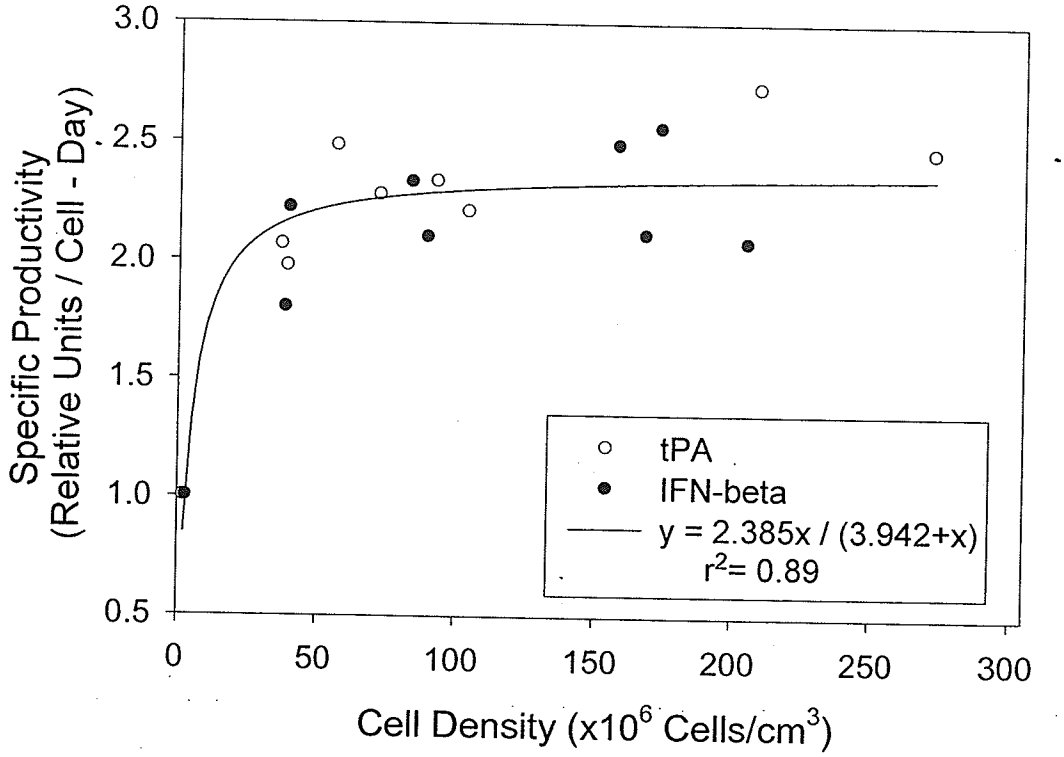

Figure 5.3: The relationship between specific productivity and cell density within Cytopore 1 microcarriers as compared to suspension cultures. The specific productivity is represented as a comparison to relative units where as the specific productivity of the suspension cultures were set to 1 unit per cell-day. The r^2 value was calculated to be 0.89.

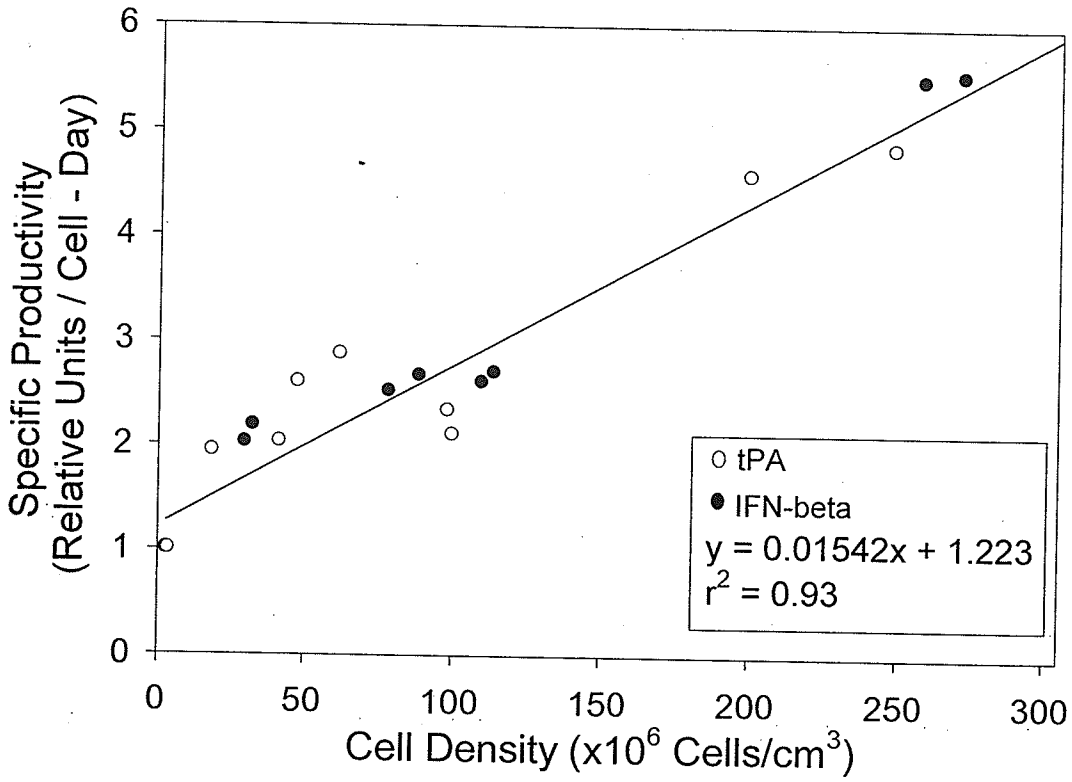

Figure 5.4: The relationship between specific productivity and cell density within Cytopore 2 microcarriers as compared to suspension cultures. The specific productivity is represented as a comparison to relative units where as the specific productivity of the suspension cultures were set to 1 unit per cell-day. The r^2 value was calculated to be 0.93.

This indicates a significant increase in Q_p for the microcarrier cultures with a cell density up to $1x10^8$ cells/mL compared to the equivalent suspension cultures. However, there was no significant evidence for a further enhancement of Q_p at higher cell densities.

On the other hand Cytopore 2 (Figure 5.4) showed a linear increase in specific productivities as the cell density within the microcarrier was increased (equation 2).

$y = 0.01542x + 1.223$ equation 2

The maximum cell density reached within the microcarriers was around $300x10^6$ cells/mL, where specific productivity was increased to over 4 to 5 times the relative units produced in suspension cultures.

5.2.3. Constant cell density within Microcarrier and its effect on Qp

The above-mentioned experiments showed that as the cell density within the microcarrier increases, the specific productivity increases. This is true up to $1x10^8$ cells/mL in Cytopore 1 microcarriers and up to $3x10^8$ cells/mL in Cytopore 2.

It is imperative to prove that the increase in specific productivity is due to the increase in the cell density within the microcarriers, as opposed to an increase in the overall cell density. Experiments were set up where a stock of microcarriers was inoculated with a cell to bead ratio of 30 cells per microcarrier. The microcarrier stock was then split such that duplicate spinner flasks were inoculated with 0.5 mg/mL, 1.0 mg/mL and 2.0 mg/mL Cytopore microcarriers. Suspension flasks were also set up as control controls.

Figure 5.5 shows the cell density of the Cytopore 1 (Panel A) and 2 (Panel B) microcarrier cultures in relation to the culture volume. Since the experiment was set up such that the cell to bead ratio was the same in each case, the overall cell density in culture medium differed depending on the concentration of microcarrier. The inoculation cell density was $0.5x10^4$ cells/mL, $1.0x10^5$ cells/mL and $2.0x10^5$ cells/mL for each microcarrier concentration, respectively.

The different cell density within each culture translates to the same cell density within each microcarrier (as shown in Figure 5.6). The cell density within each microcarrier was similar for each of the microcarrier concentrations until day 5 ($p>0.15$). After this point the cell density within the microcarrier at the higher Cytopore microcarrier concentrations appeared to plateau. This is believed to be due to nutrient limitations within the culture, as the cultures were not fed during the culture period.

The IFN-beta titers were as measured by ELISA and are shown in Figure 5.7. The titers increased as the concentration of cells per carrier increased, as well as the concentration of cells in the overall culture volume. The maximum product titre for Cytopore 1 microcarriers (Panel A) was measured as $16.8x10^6$ Units/mL from 2.0 mg/mL, $8x10^6$ units/mL for 1.0 mg/mL and just over $6x10^6$ units/mL for 0.5mg/mL. This was a similar case with Cytopore 2 microcarriers (Panel B), where the maximum protein titre was $14.2x10^6$ units/mL from 2 mg/mL, $7.5x10^6$ units/mL from 1 mg/mL and $4.1x10^6$ units/mL from 0.5 mg/mL.

Figure 5.8 shows the comparison between the specific productivities at the varying Cytopore 1 and 2 concentrations. The calculated specific productivities of the varying

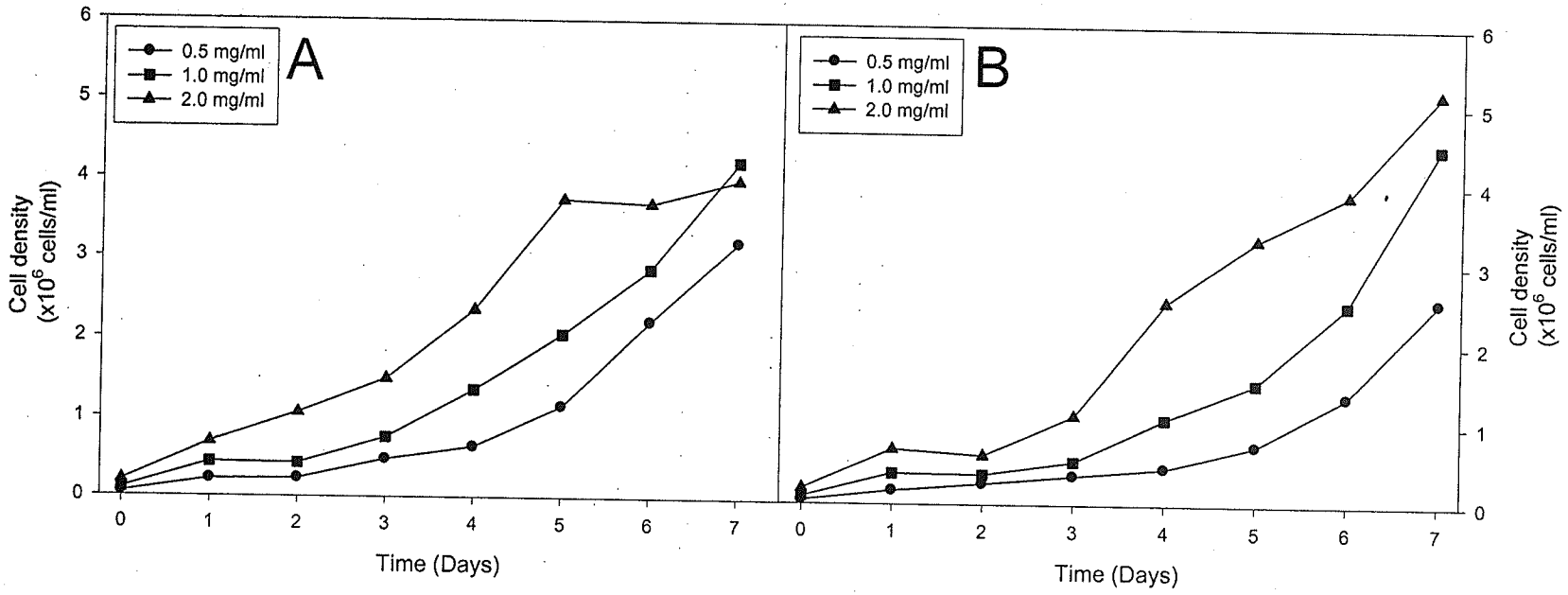

Figure 5.5 Cell density of IFN-beta producing CHO cells in Cytopore 1 (A) and Cytopore 2 cultures (B) in 100 ml spinner cultures. The Cytopore cultures were inoculated with approximately 30 cells/microcarrier bead where ● = 0.5 mg/mL, ■ = 1.0 mg/mL and ▲ = 2.0 mg/mL Cytopore microcarriers. The values are represented as means of duplicate samples.

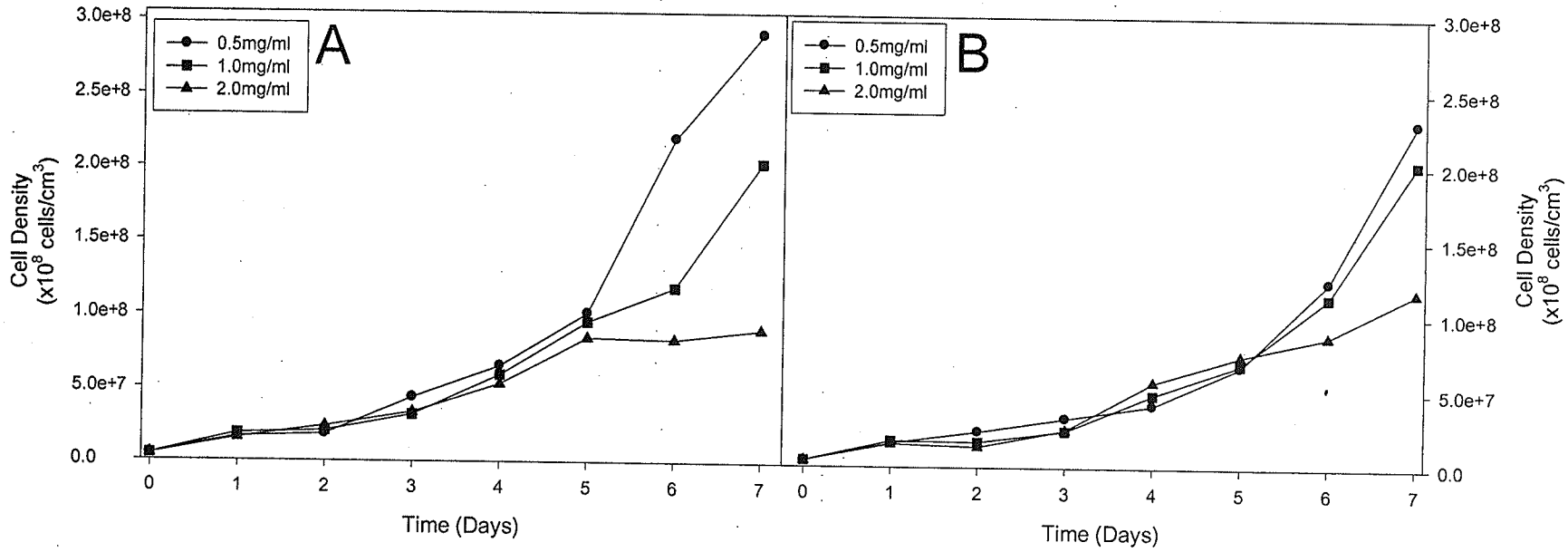

Figure 5.6 Cell density of IFN-beta producing CHO cells within Cytopore 1 (A) and Cytopore 2 (B) microcarriers where ● = 0.5 mg/mL, ■ = 1.0 mg/mL and ▲ = 2.0 mg/mL. The initial inoculum was 30 cells/bead. The values are represented as means of duplicate samples.

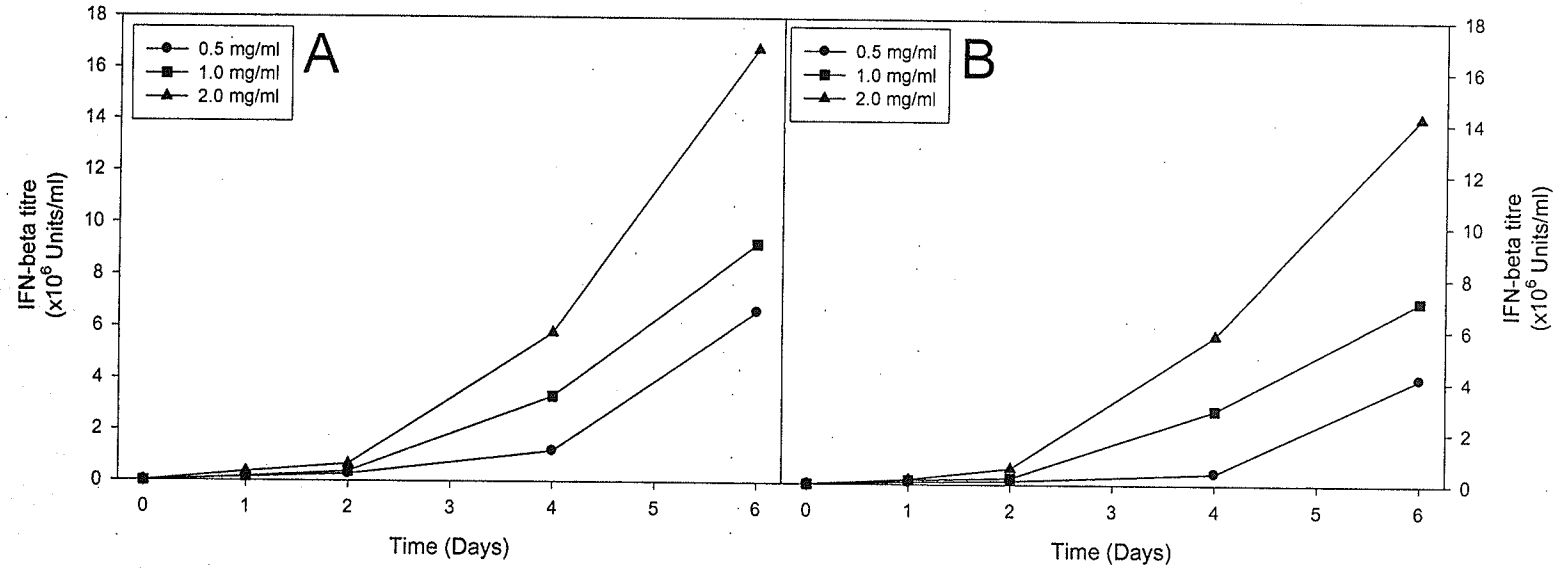

Figure 5.7: Denatured IFN-beta titers as quantified by ELISA from Cytopore 1 (A) and Cytopore 2 (B) cultures. The cultures were inoculated at 30 cells/bead. The various microcarrier concentrations tested were 0.5 mg/mL (●), 1.0 mg/mL (■) and 2.0 mg/mL (▲) Cytopore microcarriers. The values are represented as means of duplicate samples.

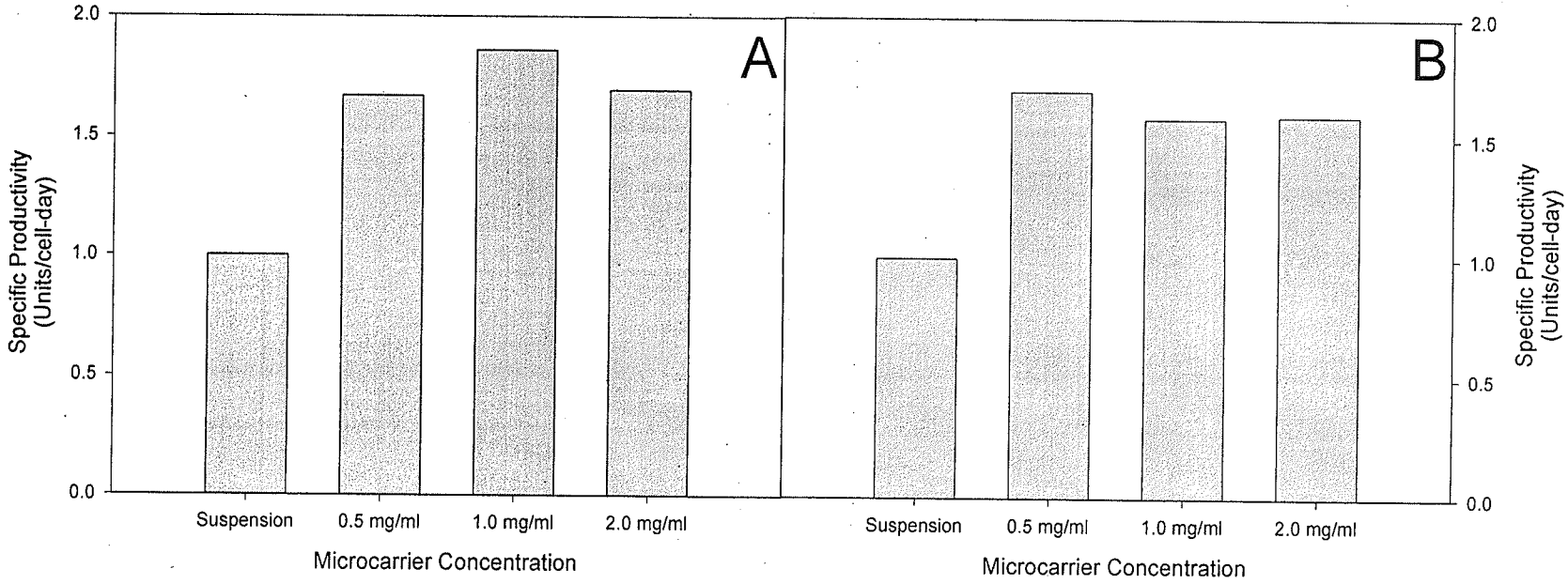

Figure 5.8: Specific productivities of IFN-beta from CHO cells grown in Cytopore 1 (A) and Cytopore 2 (B) microcarrier cultures. The values are represented as means of duplicate samples

concentrations of Cytopore are constant, as are the cell densities within the microcarriers during the exponential phase of the culture. However, it is recognized that the specific productivity is higher in the Cytopore cultures compared to the suspension cultures even though the overall cell density is similar. From this experiment, it is evident that the specific productivity increase is dependent upon the cell density within the microcarrier as opposed to the overall cell density.

This is further explained by comparing the specific productivity of a higher density culture. When Cytopore 1 microcarriers are inoculated such that the cell to bead count is 150 cells/microcarrier and monitored over the culture, the specific productivity is calculated to be 2.1 units/cell/day. The calculated specific productivity is higher in the lower density cultures that were inoculated at a fifth of the cell concentration; however the specific productivity is the same in both 0.5 mg/mL and the 1.0 mg/mL Cytopore microcarrier cultures.

5.3. Discussion:

The results reported here show that further enhancement in protein productivity can be made by increasing the effective density of cells by entrapment within the matrix of a macroporous carrier. The Cytopore macroporous carriers used in this study allow for the entrapment of CHO cells in an environment in which tissue-like high densities ($>10^8$ cells/mL) are obtained.

The specific productivity was enhanced for both cell lines when grown in the microcarrier cultures. There was a 5-fold increase in specific productivity by both CHO

cell lines when cultured in Cytopore 2 and a 2.5-fold increase in Cytopore 1. Although there appeared to be no difference in the profiles obtained for the two cell lines, the use of Cytopore 2 carriers resulted in significantly higher productivity values with a very close correlation between specific productivity and cell density within the carriers (Figure 5.4).

The entrapment of cells in various microcarriers has been shown previously to increase the productivity of cell-secreted proteins. Cytoline is a weighted macroporous microcarrier composed of polyethylene beads and was used in a fluidized bed bioreactor (Amersham 2002c). Using such a culture system, Kong *et al.* (1999) reported a 5.5-fold increase in specific productivity for a recombinant osteoblast-derived antiviral protein (OAP) from CHO cells as compared to a stirred tank bioreactor culture using Cytodex microcarriers. The entrapped cells in this system attained a maximum density of 5.3 x 10^7 cells/mL. Using a similar Cytoline culture system, we have reported from our laboratory a greater than two-fold increase in specific productivity of recombinant human erythropoietin (EPO) from CHO cells when compared to a suspension culture (Wang *et al.*, 2002). In both cases, the increase in specific productivity was attributed to the higher cell densities attained within the entrapped space of the microcarriers. Durrschmid *et al.* (2003) reported similar specific productivities of human Arylsulfatase B enzyme (ASB) from CHO cells grown in the Cytoline culture system compared to a suspension perfusion system. However, in this case similar high densities (>10^7 cells/mL) were attained in each of the culture systems.

The phenomenon of enhanced productivity from entrapped cells grown to high densities in a microenvironment has been previously observed for monoclonal antibodies

secreted by murine hybridomas (Lee *et al.*, 1991) and from transfected BHK cells (Yamaguchi *et al.*, 1997). Both the specific and volumetric productivity of a murine hybridoma was enhanced 3-fold following immobilization in calcium alginate beads (Lee *et al.*, 1991). They ascribed the advantages of immobilization to: (a) a lower growth rate (b) enhanced communication between cells in the close packed environment (c) decreased physical stress in the protected microenvironment and (d) production of autocrine factors that could accumulate to high concentrations in the local environment. These authors reported that the enhancement of Mab productivity was dependent upon the culture conditions with higher productivities favored in spinner flasks rather than T-flasks. The most plausible explanation offered was that the production of autocrine factors produced by cells within the microcarriers at high cell densities could be affected by the stirring conditions of the culture (Lee *et al.*, 1993).

In applying these various explanations to our results, we noted that the growth rates of all cells in the microcarrier cultures (0.57 to 0.73 day^{-1}) were significantly lower than those in free suspension (0.85 day^{-1}). The lower growth rate in the entrapped cultures could give rise to a higher proportion of cells in the G0/G1 phase of the cell cycle, which could in itself lead to higher productivity (Luo and Yang, 2004). It has been shown that cells in the G1 phase produce higher levels of recombinant protein (de Boer *et al.*, 2004). However, the range of measured growth rates of CHO cells in our experiments did not correlate directly with the range of specific productivities observed and so this could not fully explain our results.

Maurer *et al.* (1999) studied the growth of human lung tumour cells in Cultispher microcarriers and showed a decreased sensitivity of these cells to a range of chemotherapeutic agents at high cell densities. This was attributed to a multicellular contact effect. The cell contact-induced drug resistance was explained by the following possibilities: (a) formation of gap junctions between cells, (b) the deposition of an extracellular matrix and (c) enhanced ligand-receptor interaction.

Lee *et al.* (1993) attributed enhanced specific productivities of Mab to an accumulation of autocrine factors in the immobilization matrix. The accumulation of growth stimulating factors secreted by cells has been previously observed in the extracapillary space of hollow fibre bioreactors (Kidwell *et al.*, 1990) and could be attributed to factors such as interleukin-6 (IL-6), which is known to stimulate protein synthesis in lymphoid cells (Aarden, 1989; Hirano *et al.*, 1986). However, the production of IL-6 in our system is an unlikely explanation for the increased specific productivity of IFN-beta.

Our results indicate that there is a linear relationship between cell densities within the microcarrier and specific productivity of either of the recombinant proteins. For Cytopore 2 cultures, as cell concentrations within the microcarrier reached $3x10^8$ cells/mL, the specific productivity was 5 times that of a comparable suspension culture. However, with Cytopore 1 a maximum value of specific productivity was reached at a cell density of $1x10^8$ cells/mL with an enhancement of 2.5-fold compared to a suspension culture. The possible explanation for a cell concentration-dependent increase in specific productivity as observed in our Cytopore cultures is compatible with both enhanced cell-

to-cell contact as well as the increased concentration of autocrine factors within the microenvironment of the beads.

The enhancement of productivity in our culture systems was significantly greater in cultures of Cytopore 2 compared to Cytopore 1 for both cell lines. It appeared that in Cytopore 1 there was a threshold cell density above which the specific productivity did not increase. In contrast the specific productivity measured for the Cytopore 2 cultures increased linearly to the highest cell density. The physical properties of the two microcarrier types differ in that Cytopore 2 has higher charge densities. We may speculate that such a difference could alter the interaction of the cells with the microcarrier matrix and cause differential protein production.

The results of our experiments are important because they show that it is possible to enhance both specific and volumetric recombinant protein production from transfected CHO lines in a macroporous microcarrier culture system by increasing cell densities to values akin to those found in tissues *in vivo* (Palsson and Bhatia, 2003).

Chapter 6

6. Influence of Hypothermic culture conditions on productivity*

6.1. Introduction

Hypothermic conditions are classified as suboptimal temperatures at which cells grow, and this is generally considered to be below 37°C for mammalian cell types. The benefits of growing cells at a lower temperature (30°C to 35°C) has been reported by many researchers, and has been reported to enhance the specific productivity and volumetric productivity of a culture. The expression of many recombinant proteins have been reported to increase during the growth and production phases from exposure to a lower temperature.

Amongst the change in productivity, there are other changes that can occur. Transcriptome analysis of CHO cells producing EPO revealed that low culture temperature could lead to changes in gene expression in various cellular processes such as metabolism, transport, and signaling pathways (Baik *et al.,* 2006). Hypothermic culture conditions induce cells to undergo delayed apoptosis due to an increased p53 protein level which in turn leads to an increased level of p21. Complexes of p21 and a cyclin dependent kinase (cdk2) inhibit cells from entering the next stage of cell division, keeping cells in a Go/G1 phase (Kumar *et al.,* 2007; Moore *et al.,* 1997). Two cold shock proteins, CIRP and RBM3, have also been studied extensively. Both CIRP and RBM3 are involved in modulation of transcription and translation by functioning as RNA chaperones (Yoon *et al.*, 2005; Nishiyama *et al.*, 1997).

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* Contents of this chapter was included in a paper: Tharmalingam, T., Sunley, K., and Butler, M. 2008. **High yields of monomeric recombinant beta-interferon from macroporous microcarrier cultures under hypothermic conditions.** Biotechnology Progress 24(4):832-838. Work reported in the paper completed by others was omitted from this chapter.

In addition, low temperature growth has been found to decrease a cell's sensitivity to apoptogens, reduce intermolecular aggregation of heterologous protein products (Rodriguez *et al.*, 2005), as well as lowering the cellular rate of nutrient uptake. This may allow for the maintenance of high producing cells at high viability for extended time periods.

The effects of low temperature are not limited to mammalian cells. Yun *et al.*(2001) report a 2-fold increase in the overall recombinant human interleukin-3 (rhIL-3) yield after decreasing the temperature from 32°C to 19°C during the fermentation of *Streptomyces lividans*. The increase was attributed to decreased tripeptidyl aminopeptidase protease activity at the lower temperature.

This chapter focuses on the growth and productivity of CHO cells producing recombinant human IFN-beta in Cytopore 2 microcarrier bioreactor cultures, in temperature-shifted suspension cultures, and in Cytopore 2 cultures combined with a temperature shift. Our objective was to attempt to enhance protein productivity as well as to minimize the molecular aggregation of IFN-beta.

From the preliminary studies of Cytopore cultures completed in spinner flasks (Chapter 4 and 5) it was decided to further investigate the production capabilities in Cytopore 2. The ability of cells to reach a 5-fold increase in specific productivity within the Cytopore 2 microcarrier and a 2.5-fold increase in volumetric protein production was further investigated. Preliminary studies into temperature shift (Appendix B) showed that there was a higher production resulting from enhanced specific productivity and overall protein yield.

The protein investigated was IFN-beta, as aggregation is an important issue for the production of this highly hydrophobic protein. Three major determinants of aggregation are the concentration of the recombinant protein, residence time of the protein in culture, and the temperature (Rodriguez 2007). A lower temperature reduces aggregation, and therefore is beneficial in large scale protein production processes.

6.2. Results

6.2.1. Growth of CHO cells in biphasic Cytopore Spinner cultures

The growth and productivity of the IFN-beta producing CHO cell line was monitored in spinner flasks. Previous studies in our lab showed that there was an enhancement of the volumetric product of recombinant protein in suspension cultures by combining the beneficial effects of enhanced specific productivity with maximal cell growth (Rodriguez *et al.* 2005). A low temperature of 32 °C allowed a limited growth of cells. However the growth was enhanced by a subsequent shift from the standard temperature of 37 °C.

We investigated the effects of a biphasic Cytopore culture involving a shift to the lower temperature after a period of cell growth at 37 °C. The rationale was to attain a large viable cell population, which could then be induced to a high cell specific productivity at the lower temperature. We induced a temperature shift in cultures at days 2 and 3 in an attempt to determine the optimal time that would enable a sustainable high cell density and furthermore lead to a high product titre. The growth profile of these cultures was compared to those maintained at either 37 °C or 32 °C.

The growth of the cells is as shown in Figure 6.1. The day 2 and day 3 shifted cultures showed a maximum cell yield of $3.4x10^6$ cells/mL and $2.9x10^6$ cells/mL by day 14, which was close to double the cell yield of the 32°C Cytopore 2 culture (with cell yield of $1.76x10^6$ cells/mL). The 37°C Cytopore 2 culture had a maximum cell yield of $3.13x10^6$ cells/mL at the end of the culture, day 7. The 37°C culture was ended at day 7, when the cell density reached a plateau.

Table 6.1 summarizes the growth and productivity yields of the temperature-shifted Cytopore 2 spinner cultures at the end of the growth period. The specific growth rates of the shifted cultures were similar (0.0105 and 0.0100 h^{-1}) where the 32°C culture had a significantly lower specific growth rate of 0.0085 h^{-1}. Compared to the shifted cultures, the 37°C Cytopore 2 control culture reached a similar cell yield ($3.13x10^6$ cells/mL) but within half the time (7 days) with a higher specific growth rate of 0.0205 h^{-1}.

6.2.2. Productivity of IFN-beta in biphasic Cytopore Spinner cultures

The production of IFN-beta was dependent on cell density, as the 32°C sample had a maximum product titre of $3.60x10^6$ units/mL by day 12, where equivalent cultures at 37°C had a maximum product of $6.51x10^6$ units/mL at day 7 (Figure 6.2). The shifted cultures showed a maximum product of $12.60x10^6$ units/mL and $9.90x10^6$ units/mL at day 12, for day 2 and day 3 shifts, respectively. The Q_p of the day 2 shifted culture was higher than that of the other cultures (0.75 units/cell-day compared to an average of 0.48

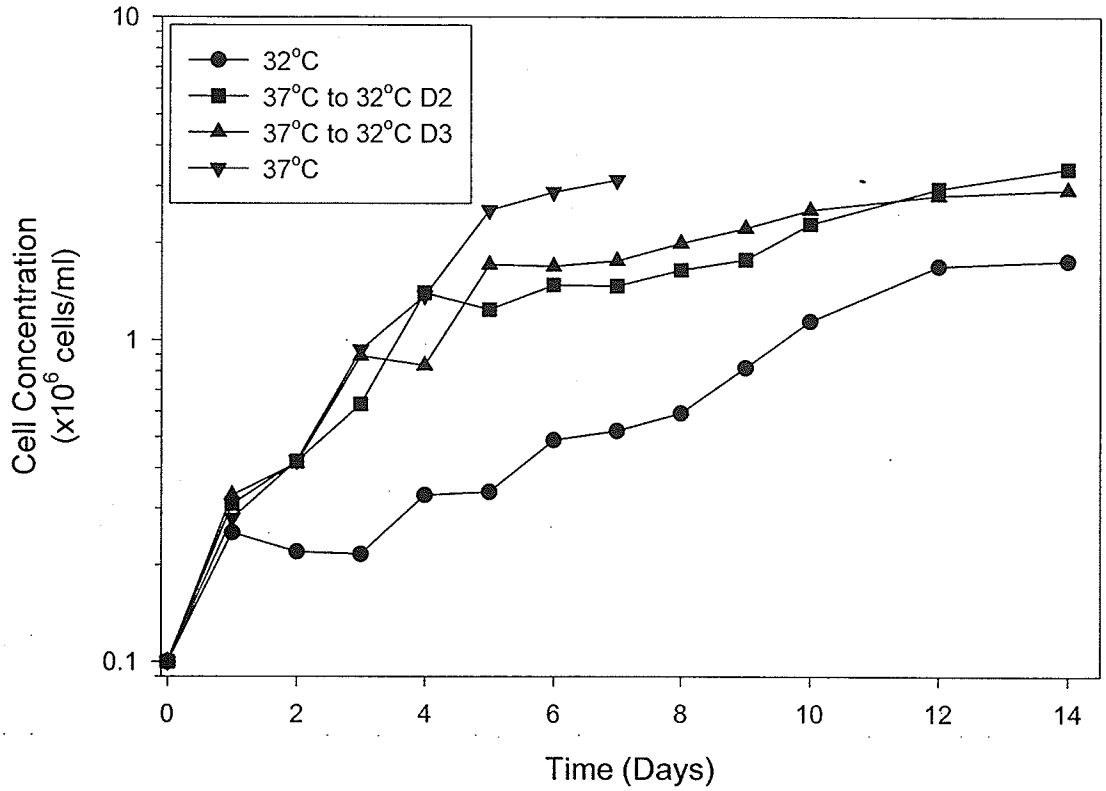


Figure 6.1: Growth profiles of CHO cells producing IFN-beta grown in Cytopore 2 cultures under temperature regulated conditions in Spinner flasks. The temperature of cultures was shifted at day 2 (■) or day 3(▲) or kept at 37°C (▼) and 32°C (●) throughout the culture period. The values represent the means of duplicate samples.

Table 6.1: Summary table from Cytopore 2 Spinner cultures.

Temperature (°C)	Day Shifted	Max. Cell Yield ($x10^6$ Cells/mL)	Specific Growth Rate (h^{-1})	Max. IFN-beta Titres ($x10^6$ units/mL)	Specific Productivity (μUnits/cell-day)	Aggregation (%)
32	-	1.76	0.0085	3.60	0.45	10.66
37-32	2	3.40	0.0105*	12.60	0.75	2.62
37-32	3	2.92	0.0100*	9.90	0.53	6.32
37	-	3.13	0.0205	6.51	0.47	35.67

* indicates the maximum specific growth rate.

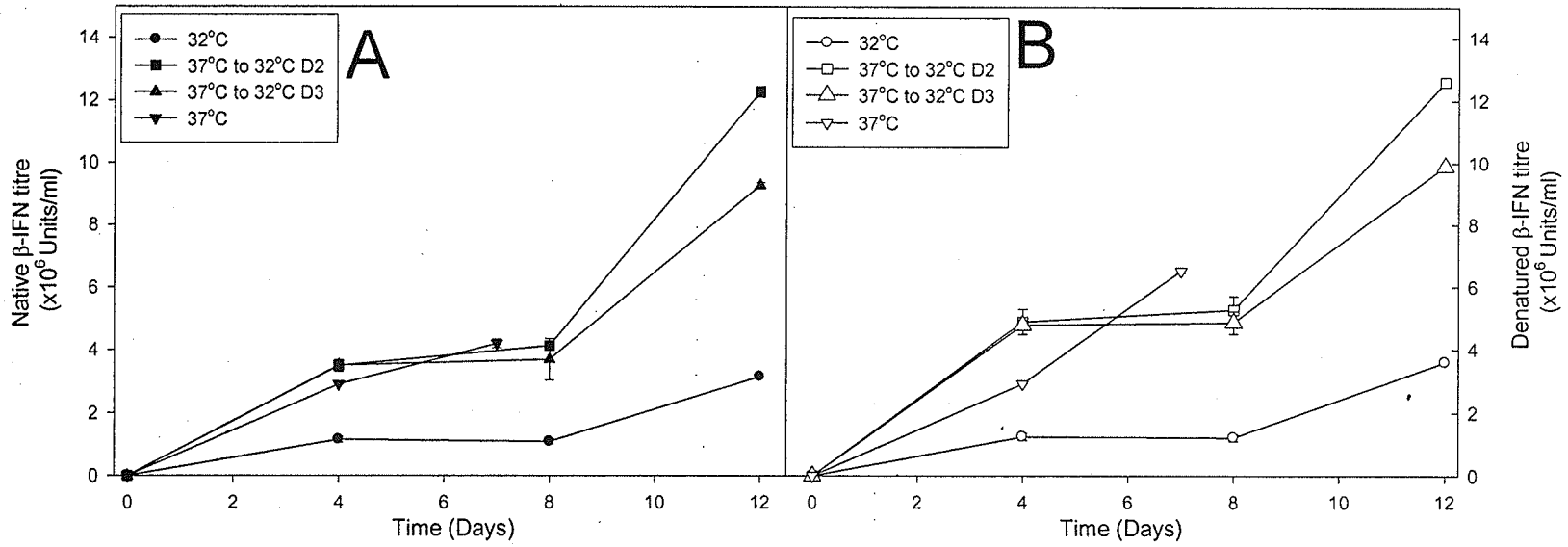


Figure 6.2: Productivity of CHO cells producing IFN-beta in spinner flasks containing Cytopore 2 microcarriers under different temperature regulated regimes. The Day 2 and Day 3 shifted cultures represent cells that underwent temperature changes from 37°C to 32°C. The values represent the means of duplicate samples ± SEM.

units/cell-day). The aggregation of IFN-beta was significantly lower for the 37°C-32°C shifted cultures and the 32°C, compared to the 37°C culture, where aggregation was greater than 3 times higher than for the other cultures (Figure 6.3).

Based on the results summarized in Table 6.1, the Day 2 shifted culture appears to show higher volumetric and specific productivity of IFN-beta, although cell yields and specific growth rates of the day 2 and day 3 temperature-shifted cultures were similar. Thus it was decided to proceed with day 2 temperature shifts in a controlled bioreactor culture.

6.2.3. Growth of CHO cells in biphasic Cytopore Bioreactor cultures

The growth of cells in suspension and Cytopore cultures exposed to variable temperatures are as shown in Figure 6.4. From our introductory experiments in spinner flasks, we were able to indentify that temperature shifts combined with Cytopore microcarriers could increase productivity of IFN-beta by over 2-fold. Thus using the results from the spinner experiments we investigated the growth and productivity of the IFN-beta CHO cell line in a scaled-up 2 L bioreactor culture.

Here we outline the growth profiles of cells cultured in suspension and on Cytopore 2 microcarriers in a 2L Applikon bioreactor either at 37°C or in a biphasic temperature-shift regimen. Based on the previous cultures in spinner flasks it was decided to decrease the temperature of the bioreactor cultures at day 2. At 37°C the suspension culture reached a maximum cell yield of 5.5×10^6 cells/mL after 5 days (Fig 6.4A). This cell density was maintained over 2 days after which the viable cell population declined. In comparison, an

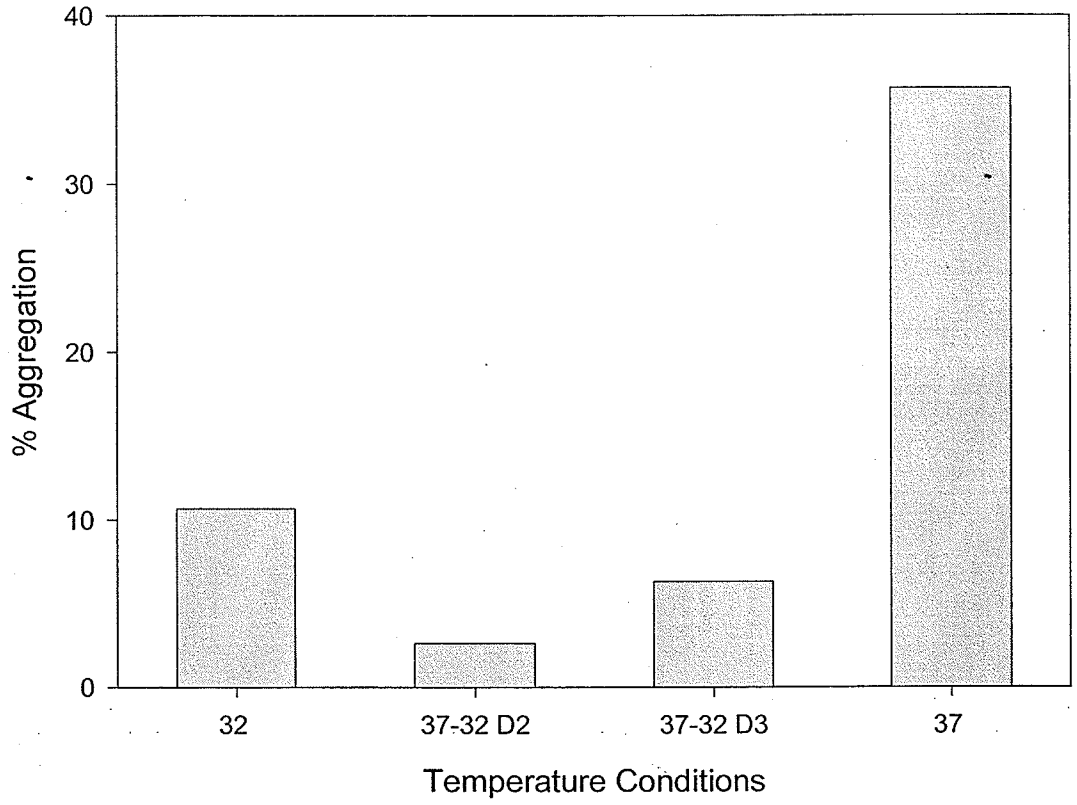


Figure 6.3: Aggregation of IFN-beta under different temperature regimes in spinner flasks. The percent aggregation was determined at the maximum protein yield in each culture. The values represent the means of duplicate samples

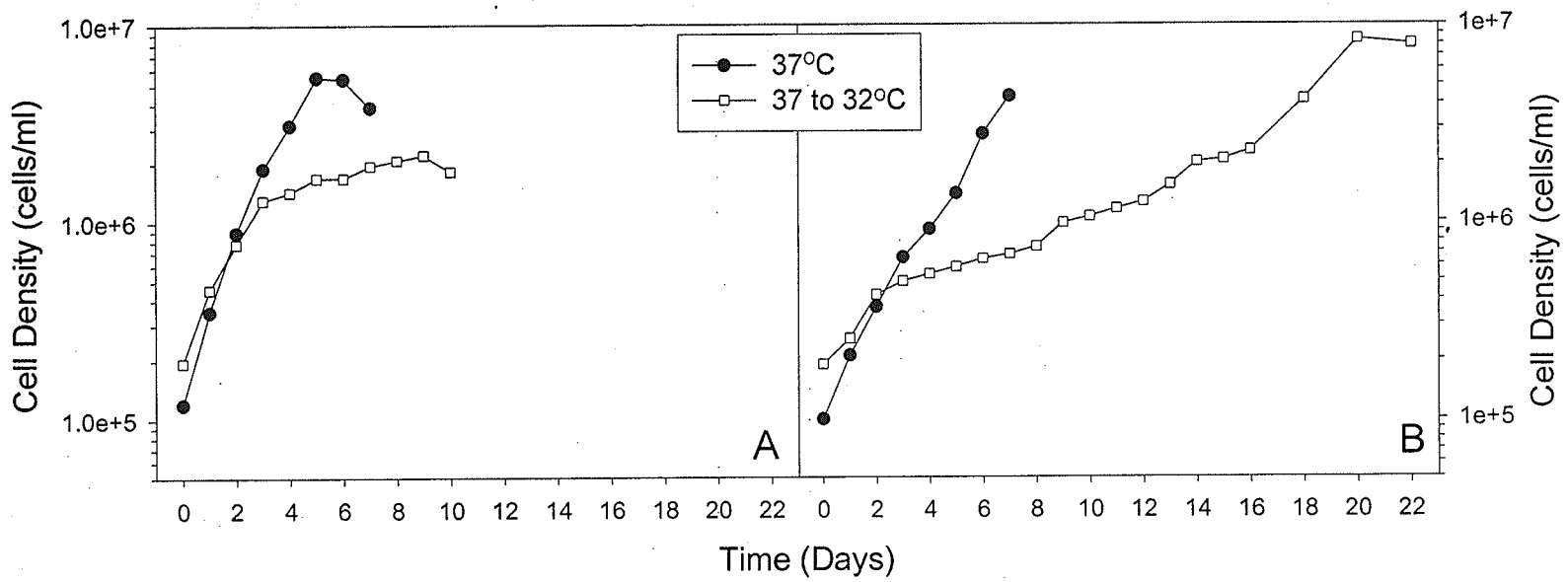


Figure 6.4: Growth in 37°C and biphasic (37°C to 32°C) cultures in suspension (A) and Cytopore 2 (B). The solid circles (●) represent single temperature cultures at 37°C and open squares (□) represent biphasic cultures. Temperature shifts were induced after 2 days at 37°C.

equivalent culture in which the temperature was decreased at day 2 yielded a maximum of $1.7x10^6$ cells/mL at day 9 after which the density decreased (by 17 %).

Growth profiles of the cells in Cytopore 2 microcarrier cultures in the bioreactor are shown in Figure 6.4B. Here there was a steady increase in cell concentration to a maximum of $4.4x10^6$ cells/mL after 7 days of culture at 37°C. After this point the cells appeared to detach from the microcarriers and grow in suspension and thus the culture was stopped. In comparison the cell concentration of the equivalent biphasic culture reached over $8.4x10^6$ cells/mL after 20 days. Only at this point did cells appear to detach from the microcarriers in the culture.

6.2.4. Productivity of IFN-beta in biphasic Cytopore Bioreactor cultures

The production of recombinant IFN-beta from these cultures is shown in Fig 6.5. The ELISA response of culture samples was measured before and after protein denaturing conditions as an indication of the degree of aggregation of the protein in culture.

The production of IFN-beta in suspension cultures is shown in Fig 6.5A. As expected the maximum yield of IFN-beta occurred at a point in the stationary phase of both cultures with a significantly higher value for the biphasic culture. The extent of protein aggregation is shown by the difference in ELISA response between the denatured and non-denatured samples. For the 37°C culture, the maximum yield from non-denatured samples was $1.63x10^6$ units/mL at day 5 after which point the values declined. The values for denatured samples ($5.41x10^6$ units/mL) were significantly higher, particularly toward the end of the culture. This showed a degree of product aggregation of up to 70

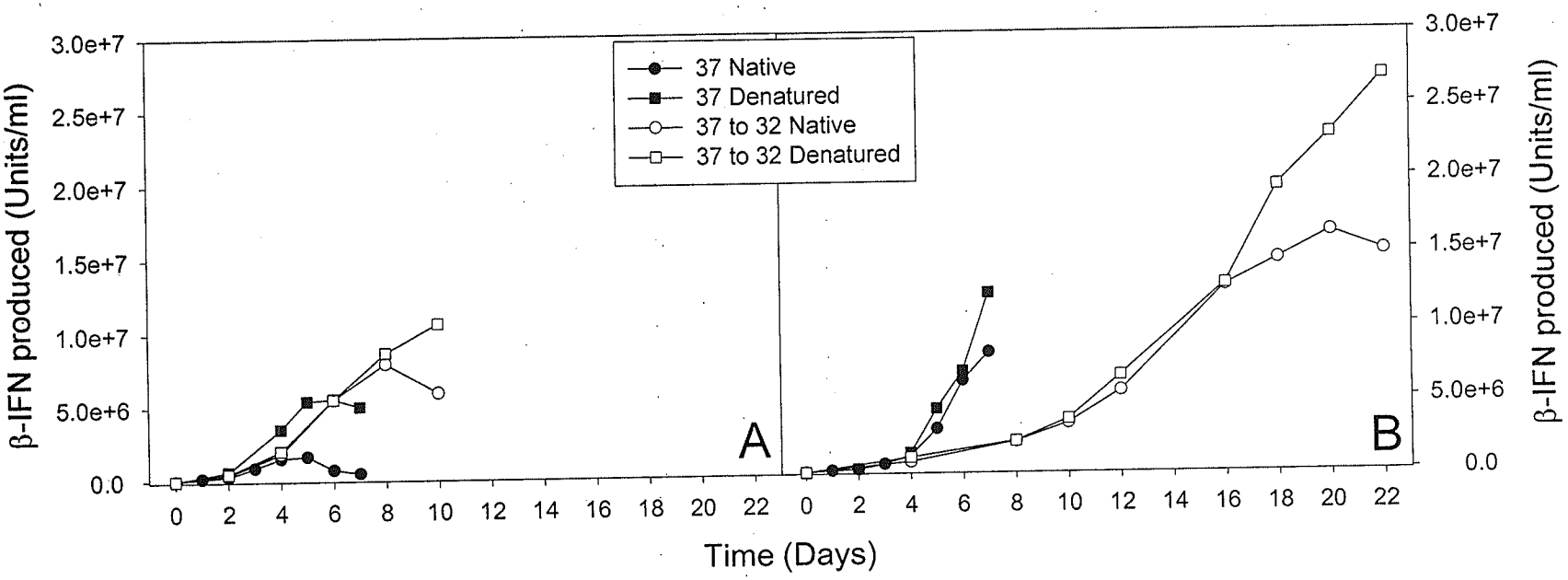


Figure 6.5: Production of IFN-beta from CHO cells grown in 37°C and biphasic (37°C to 32°C) cultures in Suspension (A) and Cytopore 2 (B). The circles represent native IFN-beta produced and squares represent denatured IFN-beta produced. Closed symbols represent 37°C cultures and open symbols represent biphasic cultures. Temperature shifts were induced after 2 days at 37°C.

%. The biphasic temperature shift of 37°C to 32°C at day 2 had a significant effect on product yields. At day 8 the yield of IFN-beta was $8.6x10^6$ units/mL with a minimal degree of aggregation (8 %), as suggested by similar responses from non-denatured and denatured samples. The denatured samples showed a maximum yield of IFN-beta of $10.6x10^6$ units/mL at day 10, although the degree of protein aggregation appeared to increase to around 40 % at this point.

The production of IFN-beta in Cytopore 2 microcarrier cultures is shown in Fig 6.5B. At 37°C, the volumetric production of native IFN-beta in the microcarrier culture ($7.9x10^6$ units/mL) was enhanced 5-fold compared to the equivalent suspension culture. The temperature shift further enhanced the production of native IFN-beta by about 9.9-fold yielding a maximum of $16.2x10^6$ units/mL by the end of the culture at day 20. In this culture there was an initial slow rate of production of IFN-beta, which followed the slow growth of the cells. Up to day 16 there appeared to be no protein aggregation but after this point the ELISA responses suggested increased aggregation up to 50 % at day 22.

Table 6.2 summarizes the results from using Cytopore 2 and hypothermic culture conditions in the bioreactor to enhance the production of IFN-beta. The product titers represent the points at which the non-denatured sample titers were at their maximum in each culture.

This shows that at these points the total product yield increased by 1.59-fold after the temperature shift regime was introduced in the suspension culture and by 2.25-fold in microcarrier cultures at the standard 37°C temperature. The enhancement in yield due to

Table 6.2: Summary table from Suspension and Cytopore 2 Bioreactor Cultures

Culture Condition	Temp (°C)	Max. Cell Yield (Cells/mL)	Avg Specific Growth Rate (h^{-1})	Qp (μUnits/ cell-day)	Max Denatured IFN-beta Titres (units/mL)	Aggregation (%)	Total Product enhancement (x-fold)
Suspension	37	5.50×10^6	0.033	0.605	5.41×10^6	70.0	1
	37-32	1.70×10^6	0.015	1.002	8.60×10^6	8.1	1.59
Cytopore	37	4.35×10^6	0.025	1.463	12.17×10^6	33.1	2.25
	37-32	8.41×10^6	0.010	0.571	22.85×10^6	29.2	4.23

both the use of microcarriers and the temperature shift was determined to be 4.23-fold which is greater than the product of the individual effects. This suggests a synergy in the enhancement of IFN-beta titers from the use of microcarriers and the temperature shift regime in these cultures.

As well as the increased overall product titre, a further advantage of these culture strategies is the decrease in aggregation of IFN-beta. Clearly, the lower temperature has a substantial effect on the degree of aggregation of IFN-beta as shown by a decrease of aggregation of 70 % to 8 % in suspension culture. The microcarriers also contribute to a lower degree of aggregation as shown by a lower percentage of 33 %. The combined effect is to reduce aggregation to 29 % but at a substantially enhanced product yield of 22.9×10^6 units/mL. This product yield could be further enhanced toward the end of the culture but at the expense of increased aggregation.

6.2.5. Nutrient Uptake of Bioreactor Cultures

The suspension culture at 37°C had a specific growth rate of $0.033 h^{-1}$ during the exponential phase from the inoculation of the culture to day 5. After day 5, the culture underwent a stationary phase, then the population slowly declined. The suspension temperature-shifted culture had a similar beginning, where the doubling time of cells was 20.6 hours during the first phase (37°C). However after the cells were shifted to 32°C, the specific growth rate declined to $0.020 h^{-1}$.

Cytopore cultures had a decreased growth rate, where Cytopore 2 cultures held at 37°C had a specific growth rate of $0.023 h^{-1}$, with a doubling time of 31 hours. The

temperature-shifted culture showed a biphasic growth pattern, where initially the growth rate was 0.030 h^{-1}, and then declined to 0.010h^{-1} for the second phase of growth (after the temperature was shifted).

Although Figure 6.4 shows the growth of each culture, there was question as to whether the cells of the temperature-shifted Cytopore 2 culture were metabolically active throughout the culture period of 22 days. In order to determine this a glucose uptake assay was performed to determine if cells were indeed metabolically active throughout the culture period (Figure 6.6).

The specific glucose uptake rates correlated with the specific growth rates, where 37°C Suspension and Cytopore cultures showed a similar specific glucose uptake rate of 2.74 mol/cell-day and 2.56 mol/cell-day, respectively. The cells in the temperature-shifted cultures had a lower specific glucose uptake rate, where the cells in the suspension bioreactor took up 1.29 mol/cell-day and cells in Cytopore 2 bioreactor culture utilized 0.65 mol/cell-day for the duration of the culture. The glucose uptake in the temperature-shifted Cytopore 2 culture was constant throughout the culture showing that there was a steady growth throughout the 22 day period.

The glutamine uptake was also measured throughout the culture period and shown in Figure 6.7. In both 37°C (Suspension and Cytopore) cultures, the glutamine uptake increased during the exponential phase, and thus was high during this period. The glutamine concentration remaining at the end of both of the 37°C cultures was low compared to the temperature-shifted cultures. At the end of the temperature-shifted cultures there was roughly 1.5 mM glutamine remaining in the culture, compared to the

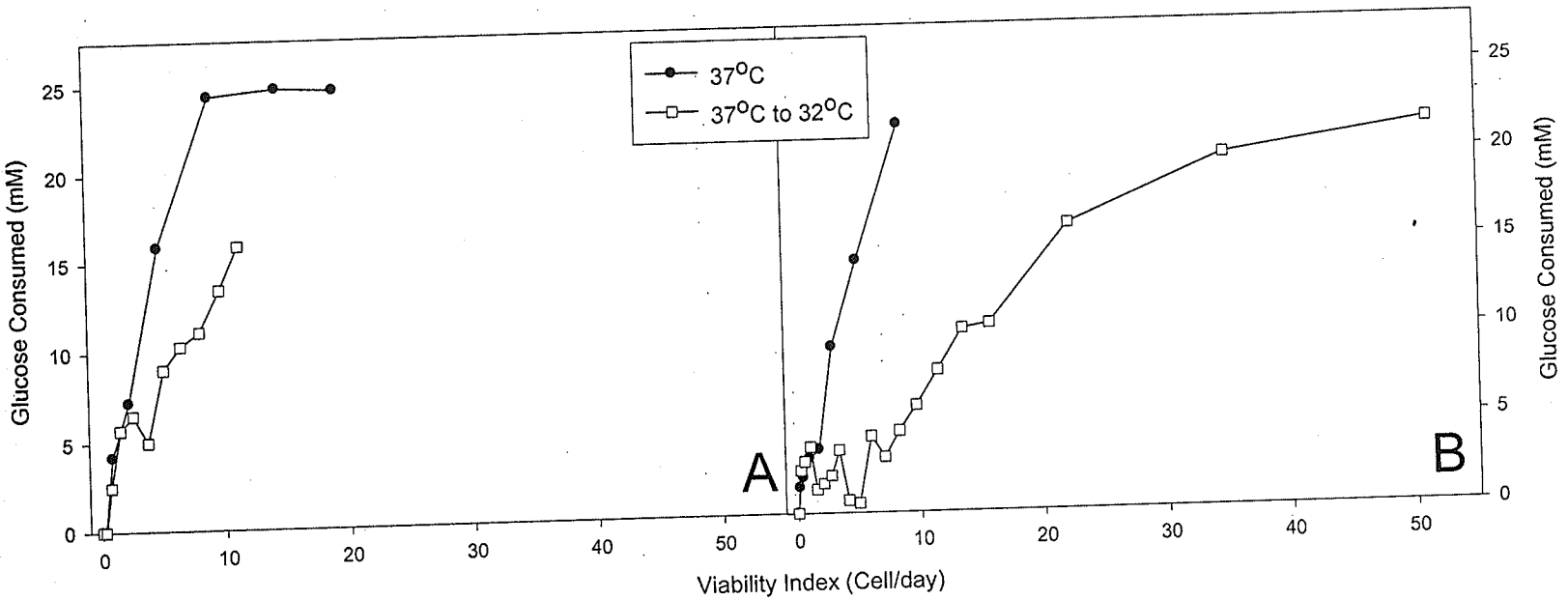


Figure 6.6: Glucose uptake over time of CHO cells producing IFN-beta grown in 37°C (●) and biphasic (37°C to 32°C) (□) cultures in Suspension (A) and Cytopore 2 (B).

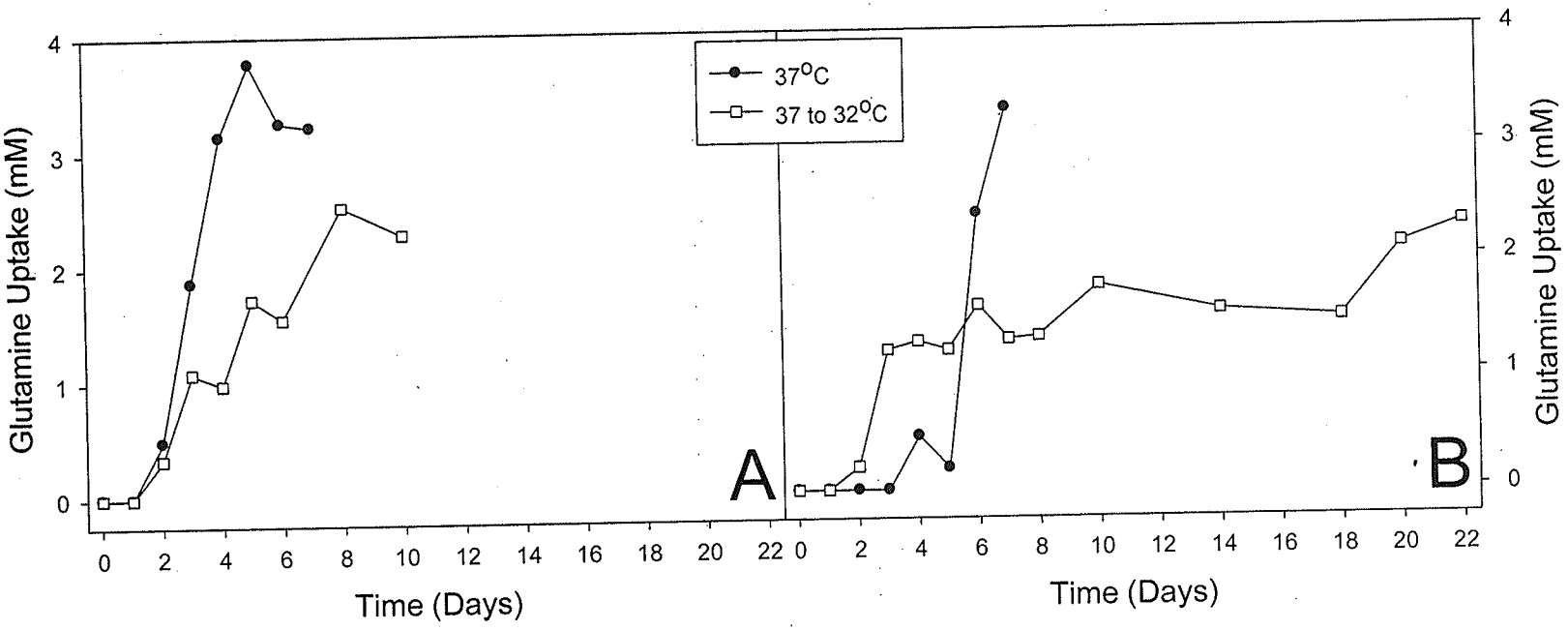


Figure 6.7: Glutamine consumed over time of CHO cells producing IFN-beta grown in (●) and biphasic (37°C to 32°C) (□) cultures in Suspension (A) and Cytopore 2 (B).

37°C culture where glutamine was totally depleted. The maximum glutamine uptake rates ranged from 0.42 to 0.74 mM/cell-day; however in the temperature-shifted cultures, the rates drop to 0.23 mM/cell-day in the suspension temperature-shifted culture and 0.02 mM/cell-day in the Cytopore temperature-shifted culture after day 3 (Table 6.3).

6.3. Discussion

The use of Cytopore microcarriers to increase recombinant protein production in small scale spinner cultures has been shown in Chapters 4 and 5. In the present chapter we compared the growth and productivity of cells in a bench-top bioreactor with Cytopore 2 or suspension cultures. The production of the IFN-beta was then compared to that of temperature-shifted suspension and Cytopore 2 cultures.

Initially the cultures were run in spinner flasks, to see if there was an opportunity for increased recombinant protein production from the cells under low temperature conditions. Previous results gathered in the lab showed an increased productivity in suspension cultures that were exposed to a temperature-shift regime (Appendix B). The preliminary studies in Cytopore 2 containing spinner flasks showed that IFN-beta expression can be enhanced by 1.9-fold volumetrically in a day 2 temperature-shifted culture compared to the 37°C culture.

Since there was an enhancement in the productivity in Cytopore 2 cultures at lower temperatures, similar to the suspension cultures, we investigated the growth and productivity of the cells in a 2L bench-top bioreactor. The use of Cytopore microcarriers and a temperature-shift regime independently showed approximately a 2-fold increase in

Table 6.3: Glucose and glutamine uptake of Cytopore 2 and temperature-shifted bioreactor cultures

Culture Condition	Temperature (°C)	Average μ (h^{-1})	Specific Glucose Uptake (pMol glu/cell-day)	Specific Glutamine Uptake (pMol gln/cell-day)
Suspension	37	0.033	2.74	0.69
	37 – 32	0.015	1.29	0.74 (days 0-3)
				0.23 (days 3 to 10)
Cytopore	37	0.025	2.56	0.42
	37 – 32	0.010	0.65	0.72 (days 0-3)
				0.02 (days 3 to 22)

total secreted recombinant protein compared to a controlled suspension culture. There was also a 5-fold enhancement in the production of monomeric, non-aggregated protein, thus indicating that these two culture methods were effective in reducing molecular aggregation of the IFN-beta in comparison to a controlled suspension culture. The combined effects of Cytopore and temperature shift showed a 4-fold increase in total protein production and an 8-fold increase in monomeric protein. Thus the combined effects of a lowered temperature and the presence of Cytopore 2 microcarriers were additive and synergistic for the enhanced production of IFN-beta.

We have shown that for Cytopore 1 and 2 microcarrier cultures there is a strong correlation between specific productivity and cell density within the microcarrier (Chapter 5). Cells grown in Cytopore 2 yield a 5-fold increase in recombinant protein specific productivity as the cell density reaches $3x10^8$ cells/cm^3. Our lab has also previously reported a 30 % increase in the titre of recombinant human IFN-beta produced in CHO cells grown in Cytopore 1 microcarriers in a bioreactor compared to a suspension culture (Spearman *et al.*, 2005).

The viability of cells appears to be extended in the cultures and this increases the amount recombinant protein obtained. Several papers show this effect for different recombinant proteins in culture systems incorporating Cytopore, for example; t-PA (Zhaolie *et al.*, 1999), u-PA (Hu *et al.*, 2000), pro-UK (Xiao *et al.*, 1999) and prothrombin (Chen *et al.*, 2001).

Cultures are traditionally maintained at 37°C; however, inducing cell growth at hypothermic culture conditions (30°C to 35°C) has been reported to enhance the specific productivity and volumetric productivity of a culture. Many studies have credited the increase in volumetric productivity to an increase in specific productivity, which under appropriate conditions can offset a lower cell growth rate (Chen *et al.*, 2004; Fogolin *et al.*, 2004b; Furukawa and Ohsuye, 1998; Shi *et al.*, 2005). Chen *et al.* (2004) reported a 40 % increase in pro-urokinase with a lowered culture temperature to 31°C. Shi *et al.* (2005) showed a 3.2-fold increase in antibody-IL-2 fusion protein production at 30°C. Fogolin *et al.* (2004a) described a 2.1-fold increase in recombinant human granulocyte macrophage colony stimulating factor.

Many theories have been formulated to explain the increase in productivity in temperature-shifted cultures. Some labs have reported a change in the cell cycle where there is an apparent shift from cells in the S phase in the cell cycle to the G0/G1 phase. Other theories include a stabilization of the mRNA of the recombinant protein in cells grown at a lower temperature (Fox *et al.*, 2004); a suppressed growth rate at lower temperatures due to the presence of a cold-inducible RNA binding protein present only at lower temperatures (Fox *et al.*, 2005); enhanced protein quality due to a lower protease activity and other temperature sensitive enzymes (Clark *et al.*, 2004).

Our study showed that Cytopore microcarriers and low temperature conditions can be used together to increase the production of IFN-beta by up to 4-fold compared to a suspension 37°C culture. Varying the temperature alone doubled total protein production and a 4.8-fold increase in monomeric protein. In comparison, Fogolin *et al.* (2004b)

reported a 2.3-fold increase in the volumetric productivity of recombinant human granulocyte macrophage colony stimulating factor with a 2 day temperature-shifted culture from 37°C to 33°C. Yoon *et al.* (2003) also indicated a 4-fold increase in the volumetric production of recombinant erythropoietin from CHO cells. An increase in the volumetric productivity was also shown by Chen *et al.* (2004), for pro-urokinase with an increase of 47 % at 34°C compared to 37°C and an associated 40 % increase in specific productivity. Fox *et al.* (2004) reported a 2-fold increase of interferon-gamma in a culture at 32°C compared to 37°C.

The increase in recombinant protein production at low temperature also appears to be cell line specific. Yoon *et al.* (2004) found that the production of an anti-4-I-BB antibody by CHO cells was not enhanced at 33°C. Furthermore, they reported that 12 parental cell lines producing the anti-4-I-BB antibody showed different levels of recombinant protein expression at low temperature. Clark *et al.* (2004) reported no difference in the production of tPA by CHO cells in 37°C and 34°C cultures.

It has been well documented that there is a decreased growth rate associated with CHO and other cells producing recombinant proteins at decreased temperature (Chen *et al.*, 2004; Yoon *et al.*, 2006; Yoon *et al.*, 2004). This decreased growth rate is explained by a shift in the cell cycle from S phase to G0/G1 phase (Chen *et al.*, 2004; Fogolin *et al.*, 2004b; Shi *et al.*, 2005). Fox *et al.* (2004) suggested that the increased specific productivity in a temperature reduced culture was due to the increased stability of IFN-γ mRNA. A cold-induced RNA binding protein (CIRP) has also been identified to suppress growth at lowered culture temperatures (Yoon *et al.* 2004). A lower temperature may also

be associated with reduced specific glucose and glutamine uptake rates and consequently reduced specific lactate and ammonia production rates (Chuppa *et al.*, 1997; Fogolin *et al.*, 2004b), also consistent with the results from this study.

The detrimental effect of a decreased growth rate on total production can be overcome by inducing a biphasic culture in which cells are grown at 37°C before the temperature is lowered for the remaining days of the culture. This temperature shift enables the initial establishment of a viable population of cells which then enter a stage of cell maintenance and enhanced production during the second phase. The balance between sufficient cell growth and enhanced specific productivity can be controlled by the appropriate timing of a temperature shift. In our cultures this balance was found empirically by a shift at day two of culture.

Cells grow within Cytopore microcarriers at a slower rate than in suspension. However, our results show that this slow rate of growth can be maintained for a prolonged period under hypothermic conditions up to 20 days compared to equivalent suspension cultures where a stationary phase was reached after 9 days. The cell densities reached in our experiments were comparable to those found in the literature (Refer to Table 6.4)

Table 6.4: Comparison of cell density data found in literature. The cell densities reported are from overall cell density in the culture and the cell density within the macroporous microcarrier.

Cell density (cells/ml culture volume)	Cell density (cells/ml bead volume)	Recombinant protein	Reference
$0.5x10^7$	$2.0x10^8$	IFN-beta	Reported here
$1.1x10^7$	$2.3x10^8$	Prothrombin	Chen *et al.* (2001)
$1.0x10^7$	$1.6x10^8$	Prourokinase	Xiao *et al.* (1999)
$1.3x10^7$	$2.2x10^8$	U-tPA	Hu *et al.* (2000)

IFN-beta is prone to aggregation in culture because of its hydrophobic characteristics. This aggregation can lead to insoluble precipitates, to the loss of activity, low bioavailability, injection site reactions, and immunogenicity (Ricci and Brems, 2004). A previous report from our lab showed that the aggregation of IFN-beta produced from cell culture is temperature-dependent (Rodriguez *et al.*, 2005). The aggregation in culture may be reduced by the addition of glycerol (Kim and Lee, 1993; Meng *et al.*, 2001; Rodriguez *et al.*, 2005; Yoshida *et al.,* 2002) or DMSO (Rodriguez *et al.*, 2005; Yoshida *et al.*, 2002). In this study we show that the presence of Cytopore microcarriers and hypothermic conditions independently decrease molecular aggregation. Furthermore the combination of these two factors decreased the degree of molecular aggregation significantly, largely due to the lower temperature but also partially due to the presence of microcarriers.

In conclusion, we have shown that the combined effects of Cytopore microcarriers and a shift to low temperature enhanced the production of recombinant human IFN-beta from CHO cells in culture. Furthermore the degree of aggregation of the isolated IFN-beta was significantly lower under these conditions.

Chapter 7

7. Glycosylation Analysis of IFN-beta from Cytopore cultures

7.1. Introduction

The glycosylation profiles obtained from proteins produced from mammalian cell culture result in a heterogeneous population of glycans. Incomplete synthesis of N-linked carbohydrate structures often leads to the heterogeneity of these structures, preventing differences in the production of consistent protein structures.

The higher production capabilities from Cytopore microcarriers and temperature shift were shown in Chapters 4, 5, and 6. The induction of higher protein yields from varying culture conditions can impact the macroheterogeneity and microheterogeneity of recombinant protein being produced. The use of Cytopore microcarriers can impact the aggregation of the protein thus affecting the intermolecular interactions between other proteins as compared to a suspension culture. Similarly the glycosylation analysis would give us an idea of the heterogeneity of the proteins resulting from different culture conditions. Thus we decided to analyse the glycans to see the impact of higher production capabilities on the heterogeneity of the glycans isolated.

7.2. Results:

7.2.1. Macroheterogeneity of IFN-beta

The analysis of the macroheterogeneity of a protein allows for the determination of the fraction of glycosylated and nonglycosylated forms of protein in relation to total protein. In the case of IFN-beta there is one N-glycosylation site at Asn-80. Culture

processes can affect the glycosylation – yielding proteins that are glycosylated and those missing glycans at Asn-80. The western blot shown in Figure 7.1 shows the two resulting banding regions, one that is identified as greater than 20 kDa (glycosylated) and the other at 18 kDa (non-glycosylated).

Densitometry analysis of western blots allowed the comparison of glycosylated and non-glycosylated IFN-beta bands. The results of the analysis are as given in Figure 7.2. At the end of the 37°C suspension and temperature-shifted suspension cultures, the densitometry values were not significantly different, where 77.13±0.75 % and 74.07±1.50 %, respectively, of the IFN-beta produced over the last 4 days of the culture was glycosylated ($p=0.12$). The IFN-beta produced in the Cytopore 2 culture yielded a significantly lower percent of glycosylated protein where 62.72±0.87 % of glycosylated IFN-beta, compared to the 37°C suspension ($p=0.00002$). However, a combination of a temperature shift with Cytopore resulted in a significantly higher percentage of glycosylated protein (84.75±0.55 %) compared to the 37°C suspension, temperature-shifted suspension, and 37°C Cytopore 2 cultures ($p<0.0002$).

The increased yield of the glycosylated IFN-beta in the temperature-shifted Cytopore 2 culture corresponded with a lower yield of the nonglycosylated IFN-beta, where less than 15 % was nonglycosylated. The ratio of glycosylated and nonglycosylated IFN-beta throughout the culture period resulted in the same ratio for over 50 % of the culture. Thus indicating that the Cytopore 2 and temperature shift was a good combination for ensuring highly glycosylated protein yield.

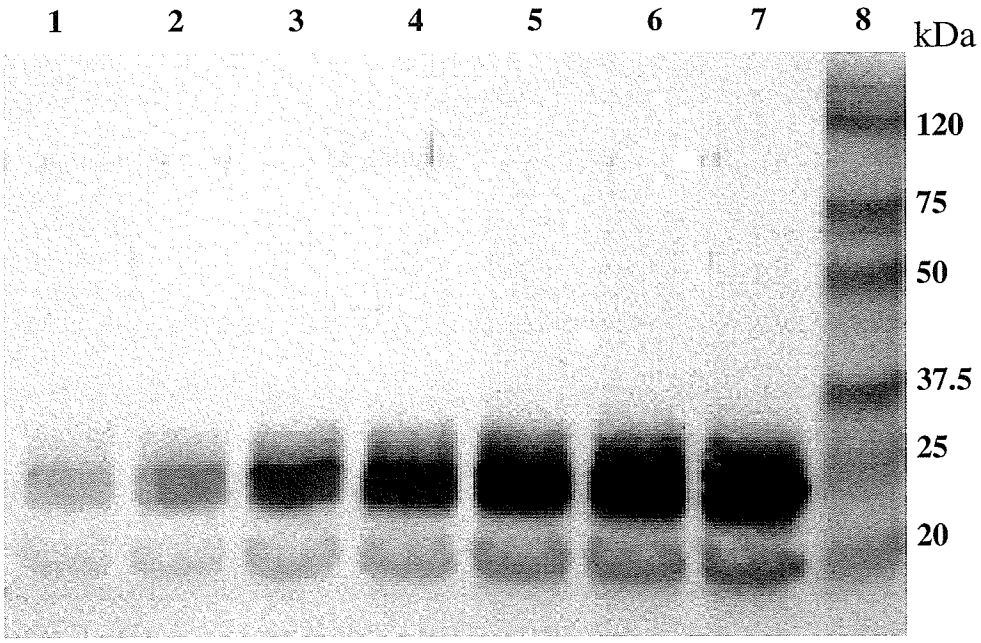


Figure 7.1 Western Blot of supernatant from an IFN-beta producing suspension culture from days 1 through 7, showing an increase in the protein intensity over time. The upper bands represent glycosylated bands and the lower bands at 18 kDa represent the nonglycosylated band. The lanes are labeled as follows: Lane 1-7, days 1-7, respectively; Lane 8, standard protein ladder.

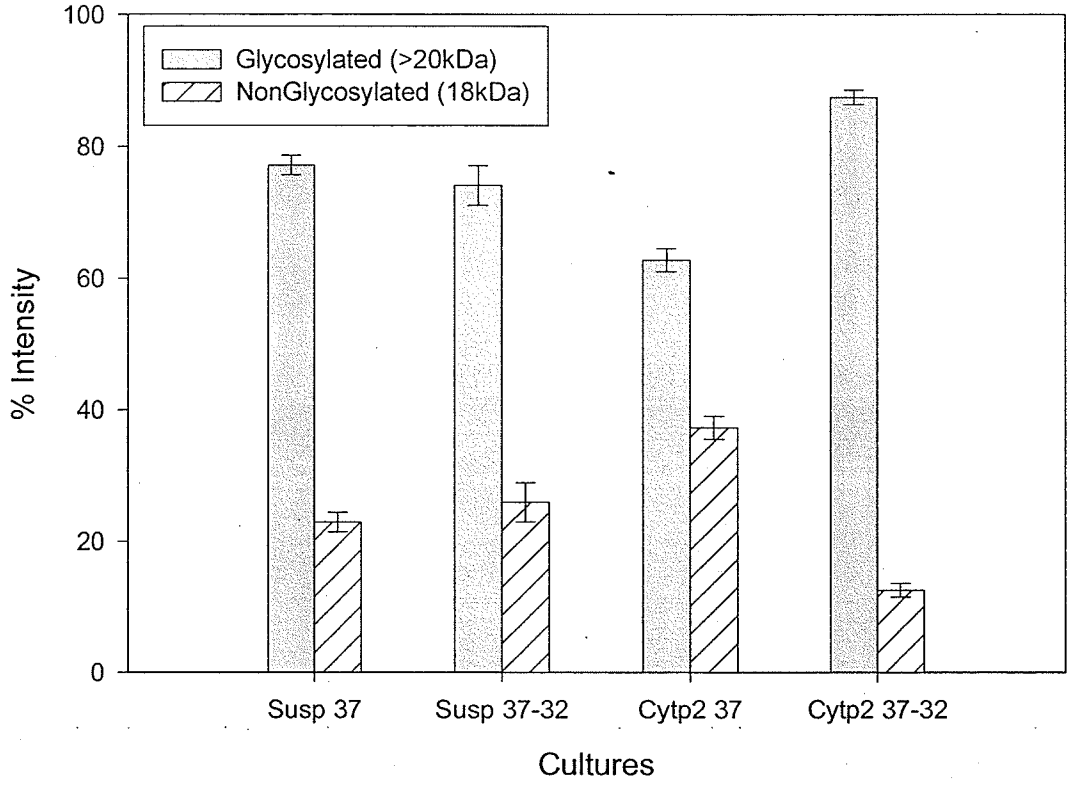


Figure 7.2: Densitometry analysis of glycosylated and nonglycosylated bands of IFN-beta from Western Blot analysis of IFN-beta averaged from the last 4 days of the culture. The grey bars represent the glycosylated bands, corresponding to the bands evident at greater than 20kDa and the hatched bars represent the nonglycosylated bands situated at 18kDa.

7.2.2. IFN-beta glycan structure confirmation

N-glycans were analysed from purified, deglycosylated, and 2-aminobenzamide (2AB) labelled IFN-beta samples (as described in Methods and Materials, Section 2.6.2). The glycan profiles were compared to a standard dextran ladder (Figure 7.3) to allow determination of the number of glucose units (GU) present within a sample. The retention times of the glycans were compared to the retention times of the standard dextran ladder to assign a glucose unit value. The assigned value could be compared to a database of known glycans structures (www.glycobase.ucd) to tentatively assign glycans structures. Structures can then be confirmed using sequential exoglycosidase digestions, the products of which can be separated by NP-HPLC and then verified by mass spectrometry (Spearman *et al.*, 2005).

Figure 7.4 shows the NP-HPLC glycosylation profile of IFN-beta obtained from a control suspension culture. The glycans were removed from the protein by in-gel digestion using PNGaseF, then isolated and labeled with 2-AB. The structures of the glycans were then confirmed using an array of exoglycosidase digestions (as described in Section 2.6.6, Materials and Methods Section) (Table 7.1). The profile B shows the glycans treated with *Clostridium perfringes* Sialidase (removal of sialic acids), followed by bovine testes β-galactosidase (galactose removal), Jack bean N-acetyl β-hexosaminidase (GlCNAc removal) and finally bovine kidney fucosidase (fucose removal) (profiles C-E, respectively).

The IFN-beta glycans show two major peaks at approximately 7.9 and 8.2 GU, which are reduced to 7.6 GU with the sialidase enzyme treatment, indicating the removal of

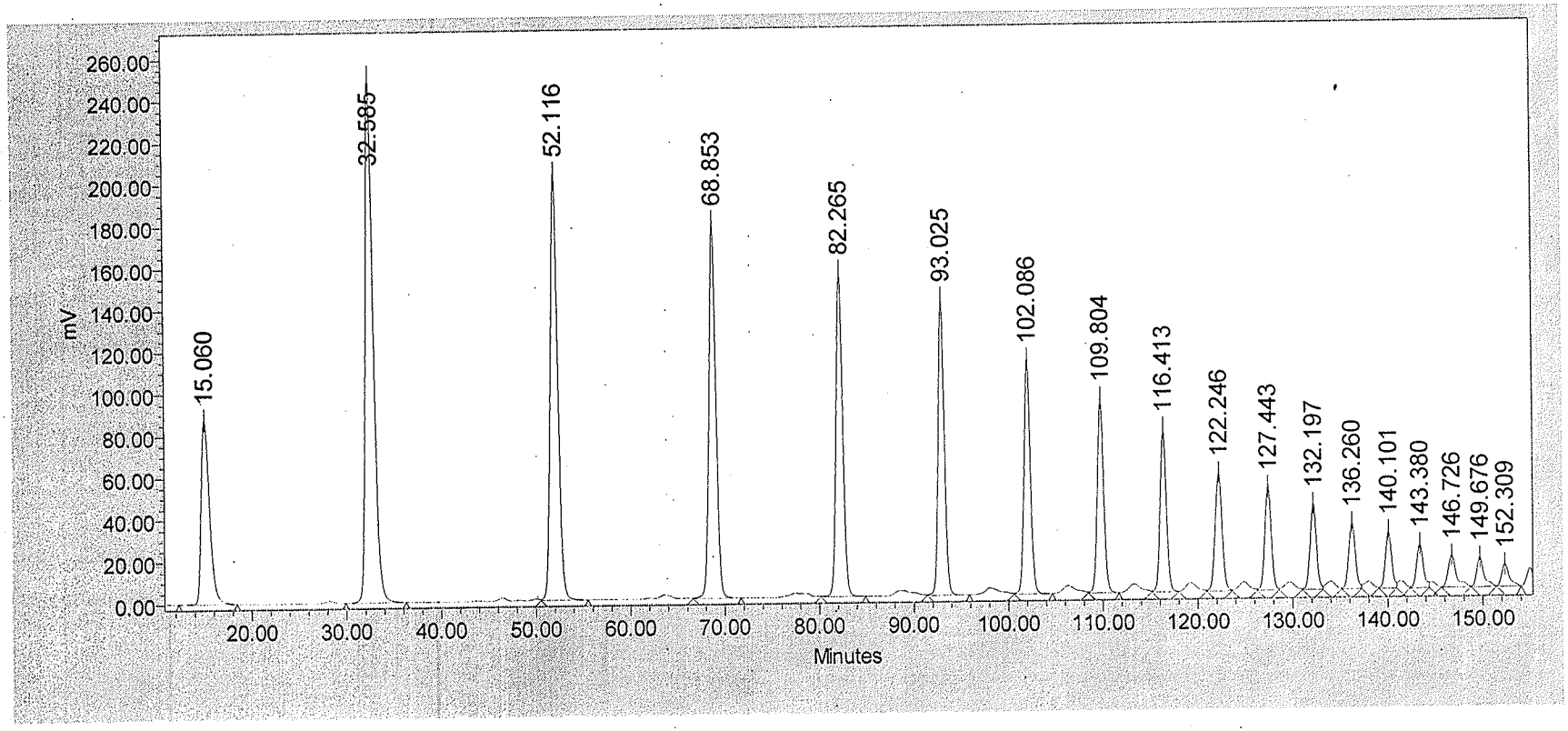


Figure 7.3: Dextran Ladder standard profile obtained from separation using NP-HPLC. Each peak represents the retention of a polysaccharide of glucose, where the first peak represents 1 glucose and the last 18 glucose units.

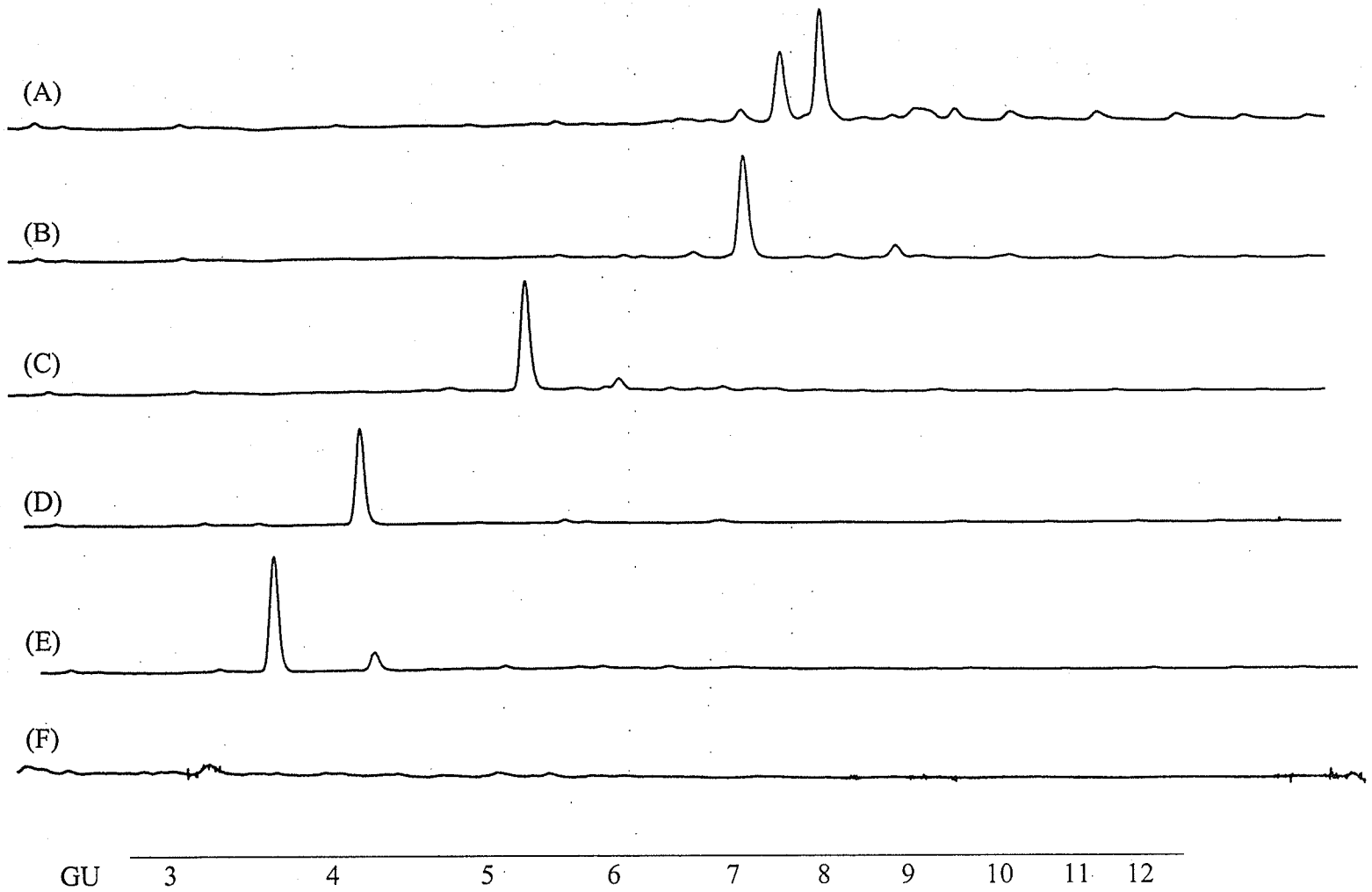
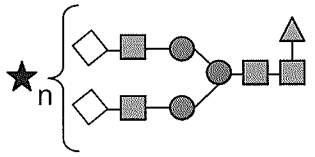
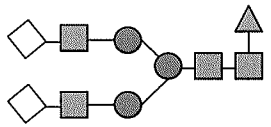
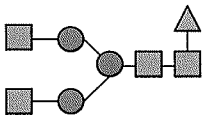
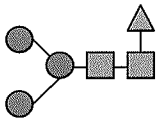
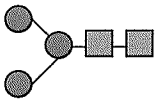


Figure 7.4: NP-HPLC Glycan profiles of 2AB labeled N-glycans from PNGase F treated Interferon-beta Exoglycosidase digestions of IFN-beta (A) with (B) sialidase, (C) Bovine Testis Galactosidase, (D) Streptococcus pneumoniae hexosaminidase, (E) bovine kidney fucosidase, and (F) a non-glycosylated profile.

Table 7.1: Exoglycosidase digestions of IFN-beta Glycans. The structures of the glycans were determined by enzymatic digestions of the terminal sugars. The core structure is termed A, with A2 referring to a biantennary glycans. Refer to figure 7.4 for glycans profiles showing the exoglycosidase digestions. The structures contain sugars including: GlcNAc (□), Man (○), Gal (◇), Fuc (△) and NeuAc (★). The Nomenclature abbreviations include A (antennae), G (galactose), F (fucose), and S (sialic acid).

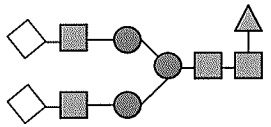
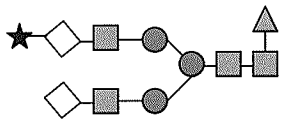
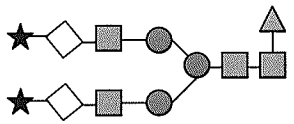
Nomenclature	Approximate Retention Time (mins)	Glucose Units	Enzyme	Proposed Glycan
A2G2FS0 A2G2FS1 A2G2FS2	105-110	7.6-8.2	N/A	
A2G2FS0	105	7.6	Sialidase	
A2F	90	5.8	Galactosidase	
$GlcNAc_2Man_3F$	78	4.8	Hexosaminidase	
$GlcNAc_2Man_3$	72	4.2	Fucosidase	

sialic acid residues on these glycans. The major peaks were assigned as the structures A2G2FS1 and A2G2FS2. These structures are shown in Table 7.2. However, there are minor amounts of triantennary and tetrantennary glycans present within the sample at GU greater than 8.3. With the addition of the galactosidase the peaks were reduced to a major peak at about 5.8 GU, which confirmed the presence of terminal galactose on the glycans. This peak was further reduced to just 4.8 GU with the addition of hexosaminidase, which cleaves off the terminal GlcNAc sugars. And finally, the removal of fucose via fucosidase digestions yielded a peak at about 4.2 GU. The final profile (F) shows a negative control of labeled non-glycosylated protein, where a sample of non-glycosylated IFN-beta was treated to PNGaseF. The confirmation of the structure using exoglycosidase digestions are important to ensure that the glycans formed are as predicted, as well to confirm the heterogeneity of the glycans profiles obtained.

7.2.3. Microheterogeneity of IFN-beta from Bioreactor Cultures

Figure 7.5 shows the HPLC profiles of derivatized glycans obtained from IFN-beta glycans from a suspension bioreactor culture. The profiles shown are for samples at days 5 (A), 6 (B), and 7 (C). The areas under the peaks were integrated (Figure 7.6) to show the relative amounts of a moiety of glycan compared to that of the overall glycans. The predominant structures identified were biantennary fucosylated structures with one (A2G2FS1) or two (A2G2FS2) terminal sialic acid residues (GU 7.9 and 8.2). Over the days of culture, the percentage of the A2G2FS1 and A2G2FS2 moieties remains constant at 75 % of the total glycans, with a ratio of approximately 1:2. Other glycan structures make up the remaining 25%.

Table: 7.2 Predominant N-glycans isolated from IFN-beta. The structures contain sugars including: GlcNAc (□), Man (○), Gal (◇), Fuc (△) and NeuAc (★). The Nomenclature abbreviations include A (antennae), G (galactose), F (fucose), and S (sialic acid).

Nomenclature	Approximate Retention Time (mins)	Glucose Units (GU)	Proposed Glycan Structures
A2G2FS0	105	7.6	
A2G2FS1	108	7.9	
A2G2FS2	110	8.2	

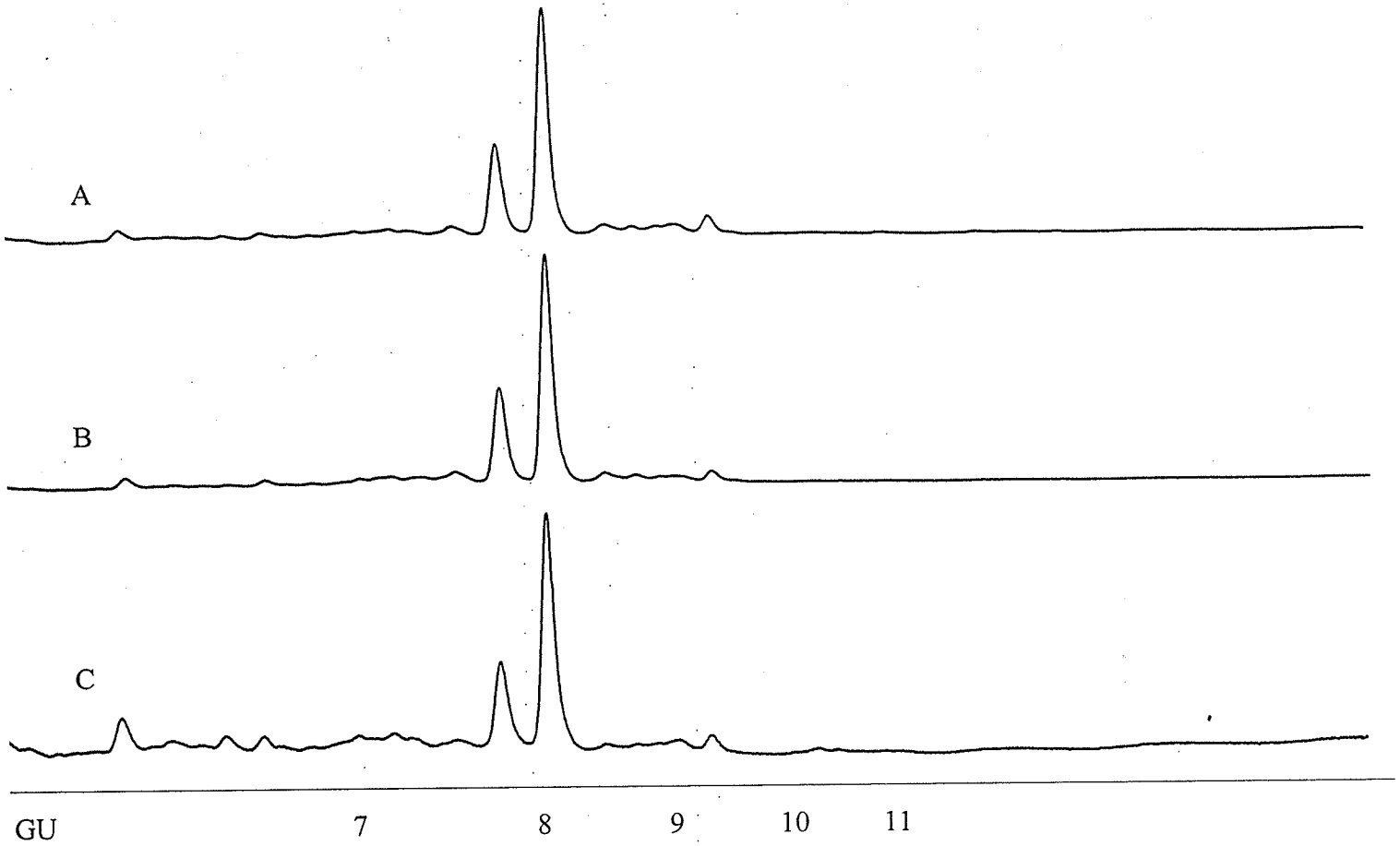


Figure 7.5: NP-HPLC Glycan profiles of 2AB labeled N-glycans from PNGase F treated Interferon-beta from Suspension Bioreactor cultures from days 5 (A), 6 (B) and 7(C).

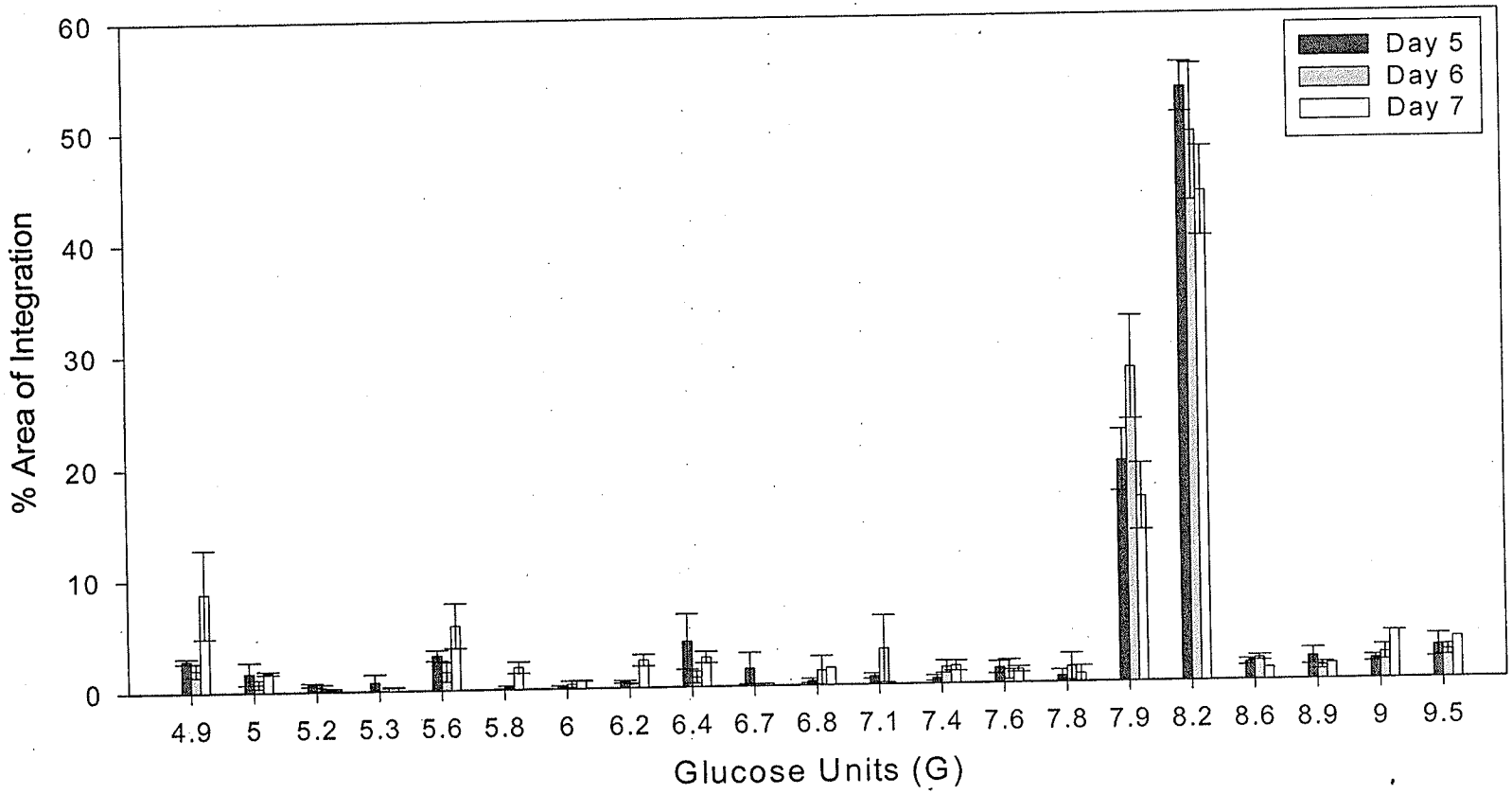


Figure 7.6: NP-HPLC analysis of IFN-beta glycans from days 5 to 7 of a control bioreactor 37°C batch culture. The two predominant glycans found on IFN-beta are biantennary fucosylated structures with one or two terminal sialic acid residues seen at GU value of 7.9 (A2FS1) and 8.2 (A2FS2). The values represent the average of 2 experiments with SEM.

The effect of Cytopore 2 microcarriers on the glycosylation of IFN-beta was determined. The glycan profiles of IFN-beta are shown in Figure 7.7, where A to D are samples from days 4 to 7, respectively. Again, the predominant moieties were A2G2FS1 and A2G2FS2 forms with a ratio of 2:3, where 25 % represents other glycan forms (Figure 7.8). The Cytopore 2 culture had a higher proportion (6-7 %) of the non-sialylated (A2G2FS0) peak, compared to the suspension 37°C culture (2-3 %) (Figure 7.6).

7.2.4. Microheterogeneity from Temperature-shifted Cultures

Figure 7.9 shows the NP-HPLC glycan profiles of temperature-shifted batch cultures. The samples were taken at days 8 and 10, and stored and purified as above. The major peaks from these profiles are the 7.9 GU and 8.3 GU representing the A2G2FS1 and A2G2FS2 structures of monosialylated to di-sialylated structures. The ratio is 5:9, where 30 % represent other species of glycans, as determined by peak integration (Figure 7.10). There appear to be glycans (4-5 %) at GU values greater than 9.5 in the temperature-shifted samples, which may be triantennary or tetraantenarry fucosylated structures with varying levels of sialic acid.

The Cytopore temperature-shifted bioreactor culture showed a larger variation of peak sizes (Figure 7.11). There was a large proportion of glycans (12 %) that are believed to have the structure A2G2FS0, as shown by the peak at 7.5 (Figure 7.12). There was also a high proportion of glycans with fewer than 7.5 GU Units (greater than 25 %) in the samples from the temperature-shifted bioreactor cultures and less than 15 % with glucose units greater than 8.3 GU. Both Cytopore bioreactor cultures (37°C batch culture and

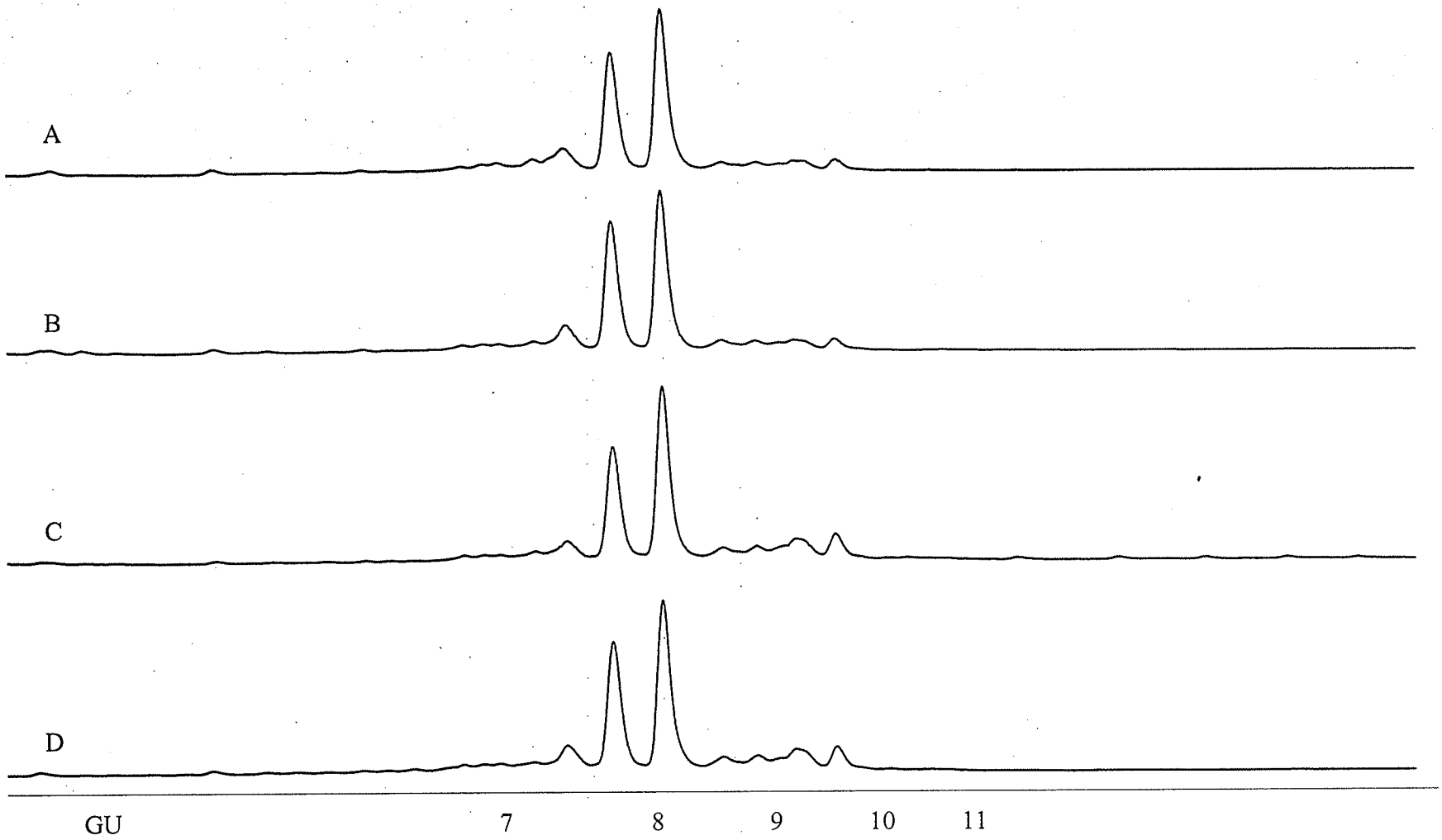


Figure 7.7: NP-HPLC Glycan profiles of 2AB labeled N-glycans from PNGase F treated Interferon-beta from Cytopore Bioreactor cultures from days 4 (A), 5(B), 6 (C) and 7(D).

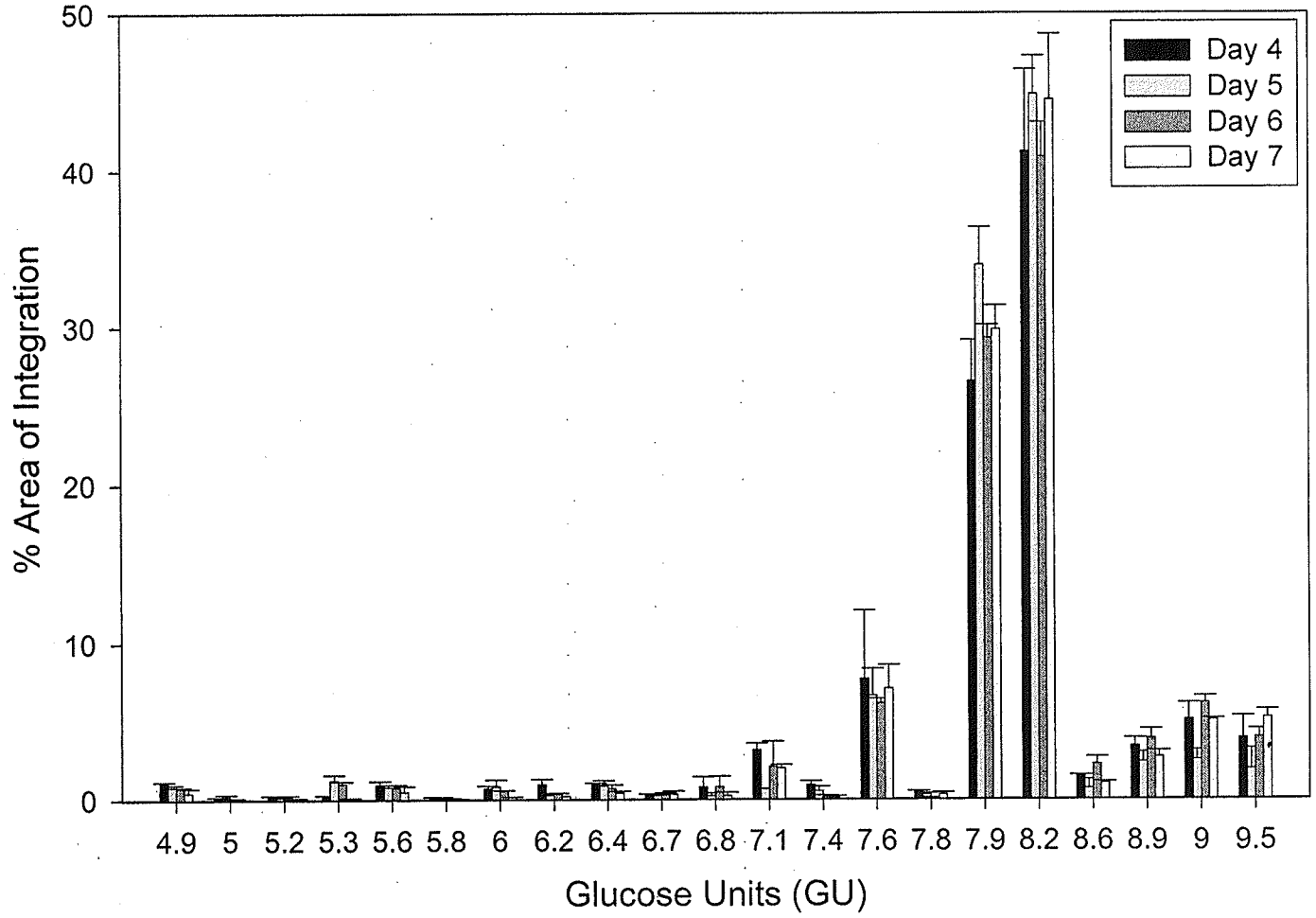


Figure 7.8: NP-HPLC analysis of IFN-beta glycans from days 4 to 7 of a Cytopore 2 bioreactor 37°C batch culture. The two predominant glycans found on IFN-beta are biantennary fucosylated structures with one or two terminal sialic acid residues seen at GU value of 7.9 (A2FS1) and 8.2 (A2FS2). The values represent the average of 2 experiments with SEM.

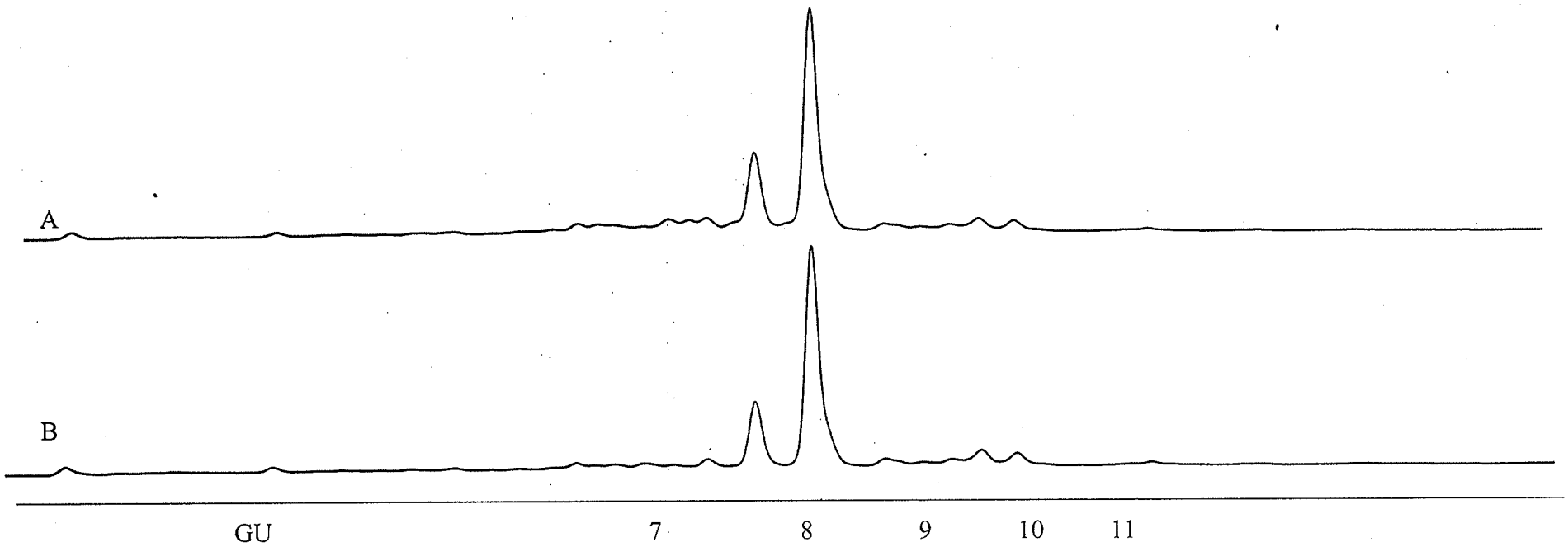


Figure 7.9: NP-HPLC Glycan profiles of 2AB labeled N-glycans from PNGase F treated Interferon-beta from Temperature-shifted Suspension Bioreactor cultures from days 8 (A) and 10(B).

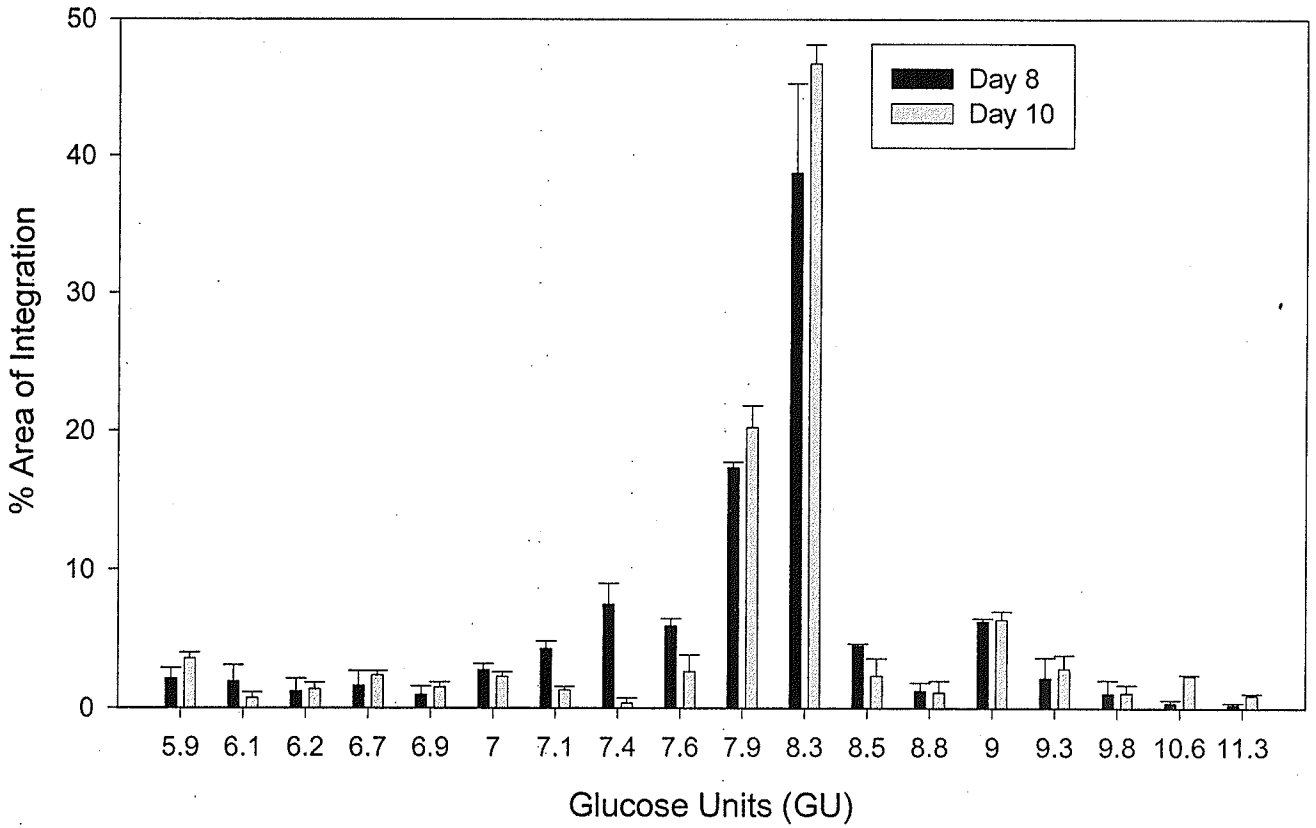


Figure 7.10: NP-HPLC analysis of IFN-beta glycans from days 8 and 10 of a temperature-shifted suspension bioreactor batch culture. The two predominant glycans found on IFN-beta are biantennary fucosylated structures with one or two terminal sialic acid residues seen at GU value of 7.9 (A2FS1) and 8.2 (A2FS2). The values represent the average of 2 experiments with SEM.

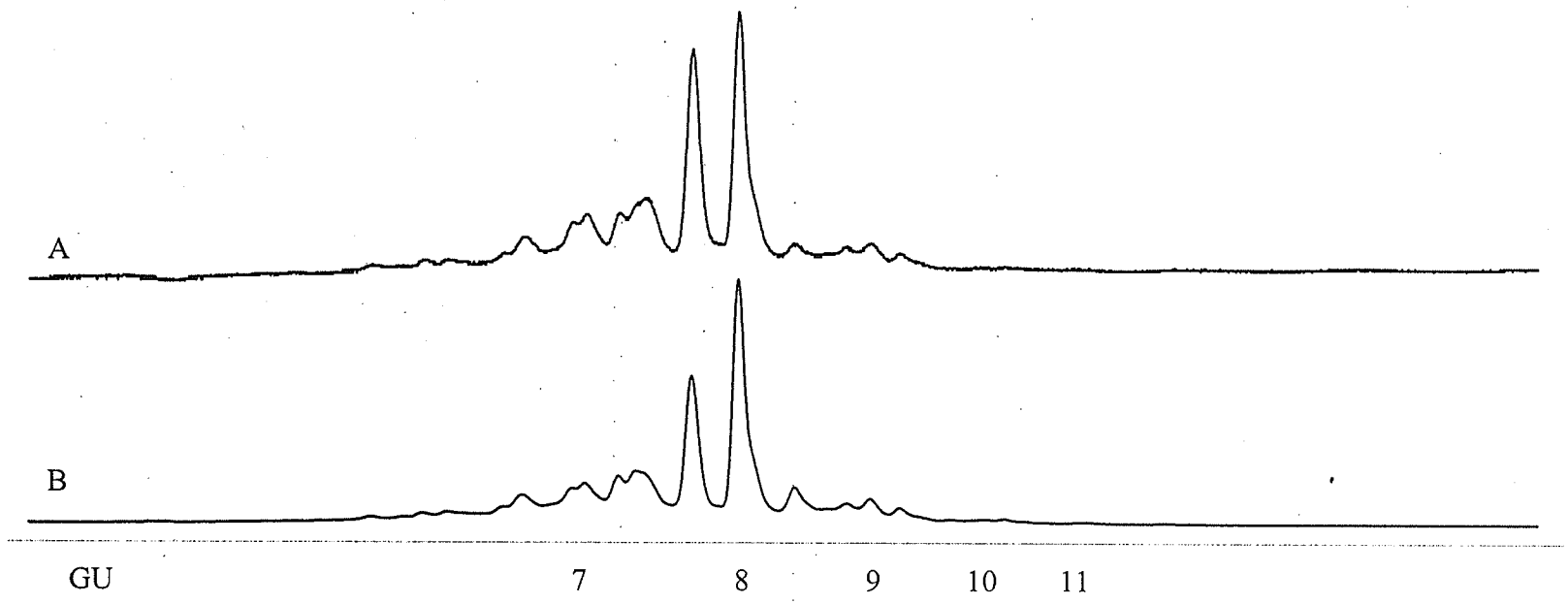


Figure 7.11: NP-HPLC Glycan profiles of 2AB labeled N-glycans from PNGase F treated Interferon-beta from Temperature-shifted Cytopore Bioreactor cultures from days 16 (A) and 22 (B).

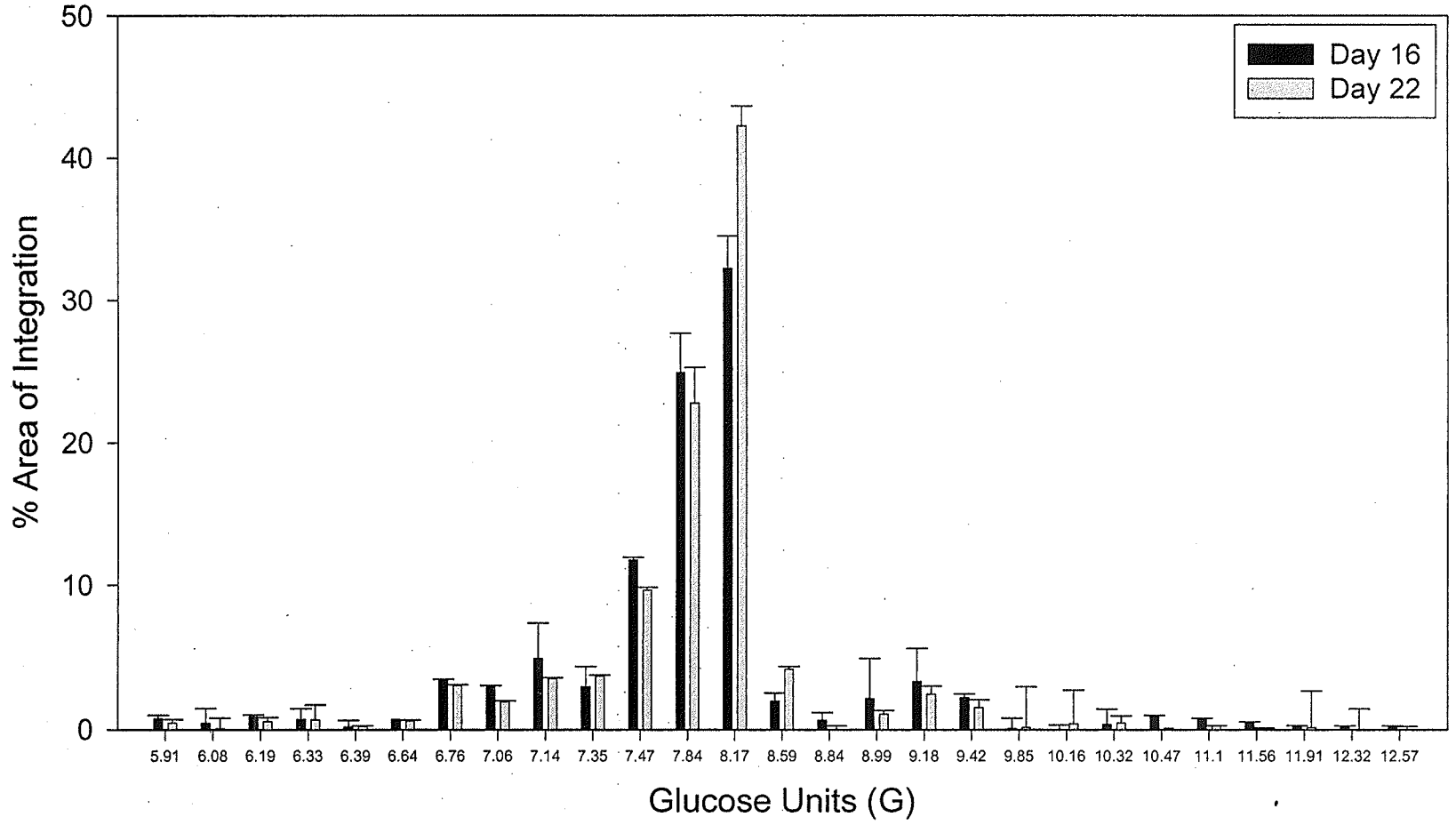


Figure 7.12: NP-HPLC analysis of IFN-beta glycans from days 16 and 22 of a temperature-shifted Cytopore 2 bioreactor batch culture. The two predominant glycans found on IFN-beta are biantennary fucosylated structures with one or two terminal sialic acid residues seen at GU value of 7.9 (A2FS1) and 8.2 (A2FS2). The values represent the average of 2 experiments with SEM.

temperature-shifted batch culture) had a high percentage (6-7 % and 9-11 %, respectively) of non-sialylated A2G2F peaks (Figures 7.5 and 7.9).

7.2.5. Comparison of Sialic Acid Contents

The major glycans isolated from IFN-beta are of the A2G2FS0, A2G2FS1, and A2G2FS2 structures. A comparison of the integration of these peaks is shown in Figure 7.13. The left panel shows the percentage of A2G2FS0, A2G2FS1, and A2G2FS2 varieties in suspension 37°C and 37 shifted to 32°C cultures. The A2G2FS0 peak comprised a minute amount of the overall quantity, where 2.9 % (37°C) and 6.5 % (37 shifted to 32°C) of the structures are of the A2G2FS0 variety. Under both temperature conditions the A2G2FS1 peak composes 29 % of the overall moiety. And the A2G2FS2 peak comprises over 65 % of the total structures. A comparison between the 37°C culture and the 37°C shifted to 32°C culture shows that the profiles are similar and the differences are insignificant ($p>0.1$).

The right panel shows the degree of sialylation of the three main peaks (A2G2FS0, A2G2FS1, and A2G2FS2) in the Cytopore 37°C and 37 shifted to 32°C cultures, where the dark bars are representative of the 37°C culture. A comparison of the A2G2FS0 peaks show that 8.4 % of the biantennary peaks were of the A2G2FS0 moiety in the 37°C Cytopore culture, compared to more than double (18.7 %) that amount in the 37-32°C shifted Cytopore culture. There was a significant difference when comparing either of the 37°C cultures to the temperature-shifted Cytopore culture ($p=0.002$ and $p=0.005$, respectively). The A2G2FS2 peak was insignificantly different when comparing the

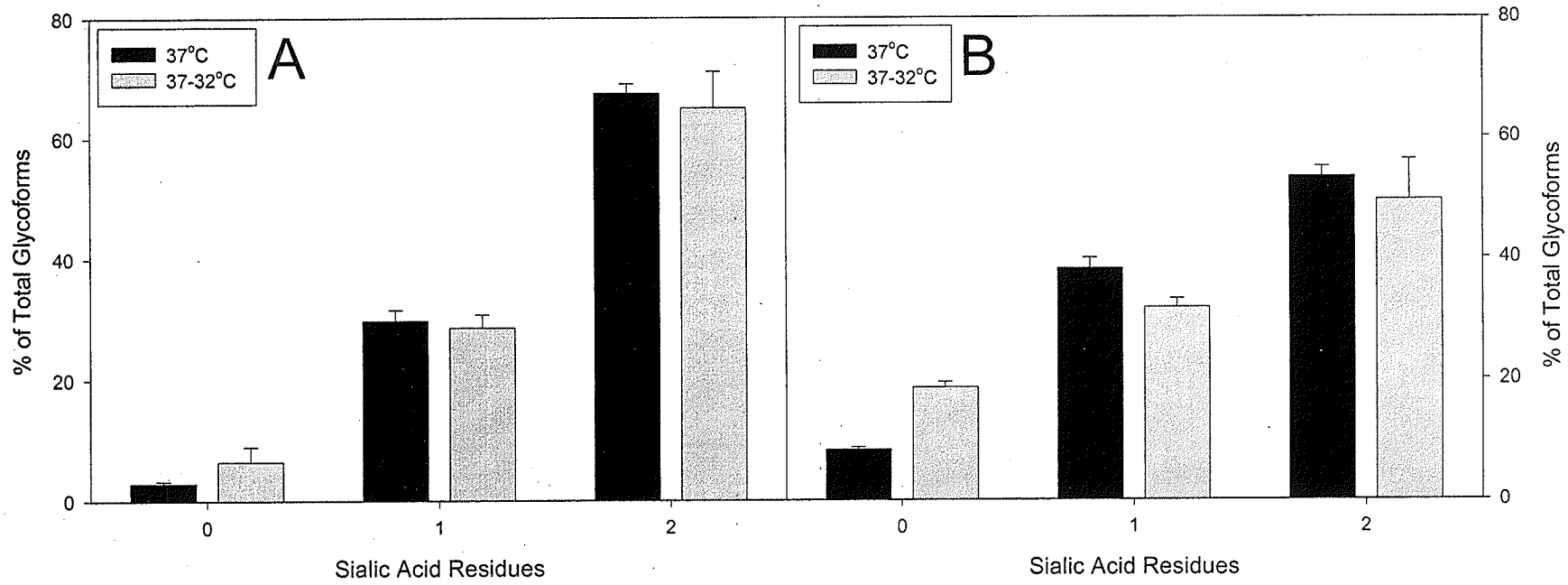


Figure 7.13: Analysis of the S0, S1 and S2 peaks (the main peaks) isolated from IFN-beta glycans isolated from Suspension cultures (A) and Cytopore cultures (B) at the end of the culture. The dark bars represent the cultures that were grown in 37°C and the grey bars represent the temperature-shifted cultures. The values are represented from duplicate sample runs on the HPLC ± SEM.

percent of the total glycoforms of the suspension cultures and the Cytopore cultures ($p>0.1$). However comparing the 37°C suspension, temperature-shifted suspension cultures to the 37°C or temperature-shifted Cytopore cultures there was a significant difference ($p<0.05$, except shifted suspension compared to shifted Cytopore where $p=0.09$).

The degree of sialylation for each culture is shown in Figure 7.14. The suspension cultures, express a similar degree of sialylation at 82.4 % and 79.1 %, for 37°C and temperature-shifted, respectively. The use of Cytopore in a 37°C culture reflects a lower degree of sialylation (72.6 %).

7.2.6. Product comparisons

Although the cultures could not be compared directly, due to the differences in sampling days, and the length of the cultures, the glycosylation analysed shows consistencies. The samples isolated from each culture were from the final days of the culture. From a production standpoint it was important to compare the final days of the production time in order to ensure a consistent product when comparing variable culturing methods. The glycan heterogeneity is consistent for all culture conditions, where there is a presence of the A2G2FS1 and A2G2FS2 peaks. Although the suspension cultures (37°C and temperature-shifted) appeared to have similar glycoform profiles, the Cytopore cultures showed a lower percentage of sialylation, and even less sialylation in the temperature- shifted Cytopore culture, where there was close to 20 % of the

A2G2FS0 moiety. The temperature-shifted Cytopore culture also had a higher percentage of the higher glycan forms (triantennary and tetraantennary).

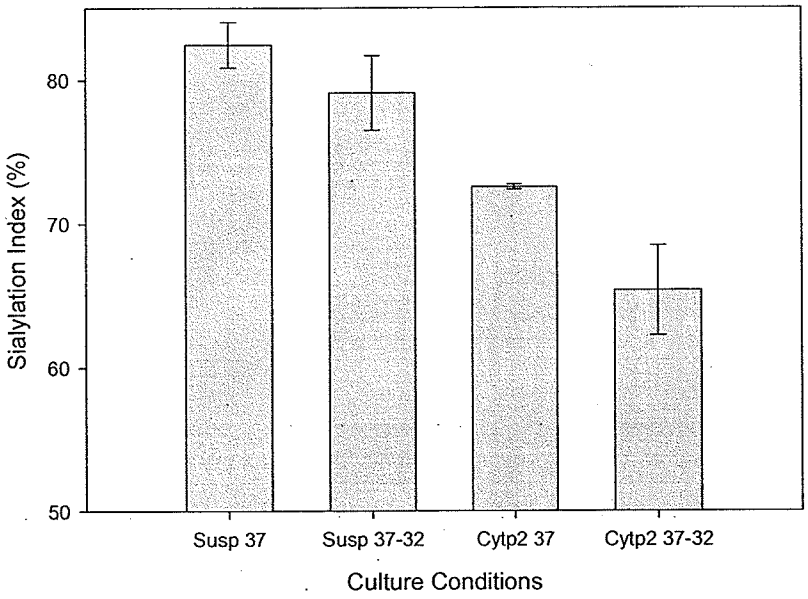


Figure 7.14: The sialylation index of IFN-beta glycans isolated from suspension and Cytopore cultures at 37°C or temperature-shifted from 37°C to 32°C. The error bars are from duplicate sample runs on the HPLC.

7.3. Discussion

The glycosylation is an important variable to monitor for the production of recombinant mammalian proteins. N-linked oligosaccharides have been shown to contribute to the solubility, pharmacokinetics, and biological activity of the glycoprotein (Ashwell and Harford, 1982; Cumming, 1991; Reju *et al.*, 2000; Sliwkowski *et al.*, 1992). It has been shown that inconsistent sialylation and galactosylation results in the variable clearance of proteins via asialoglycoprotein or mannose/GlcNAc receptor mediated endocytosis (Ashwell and Harford, 1982).

The glycosylation of IFN-beta is important for the efficiency of the protein with respect to its biological activity. Utsumi *et al.* (1995) reported decreased activity of IFN-beta produced in CHO cells on human hepatoblastoma cells compared to other cell lines that produce proteins with higher sialic acid content. The oligosaccharide moieties reported in our studies correspond with the commonly found glycans found on IFN-beta from other cell lines, where the glycans is most commonly fucosylated with one or two sialic acid residues (Conradt *et al.,* 1987; Kagawa *et al.,* 1988; Utsumi *et al.,* 1989). Conradt *et al.* (1987) reported that 95 % of glycans isolated from CHO cells producing IFN-beta were of the A2G2FS2 moiety and the remaining 5 % were of the tri- or higher antennary glycoforms. The glycoforms isolated in our case are similar; however, there is a combined total of 70-80 % of the mono-sialylated and di-sialylated glycoforms.

The macroheterogeneity is important to quantify the proportion of glycosylated protein in comparison to the nonglycosylated protein. The glycosylation has been shown to be important for proper protein folding, solubility, recognition, ligand binding, protein

stability, or it may have no function at all and be completely replaceable (Wyss and Wagner, 1996). The absence of a glycan can often lead to loss of efficacy of the recombinant protein in the treatment of a particular disease.

The increase in production of IFN-beta by the use of Cytopore and temperature shifts caused changes in the glycosylation of the proteins. The suspension cultures (37°C and temperature-shifted) led to a similar percentage of glycosylated compared to nonglycosylated protein. However, the use of Cytopore resulted in a decrease in glycosylated protein (65 %) whereas inducing a temperature shift to a Cytopore culture produced a higher quantity of glycosylated protein at 85 %.

The reasons for this increase in glycosylated protein are unclear. However, we can speculate that the lower temperature resulted in a lower metabolism, and thus lower quantities of glucose were utilized during the culture period (Chapter 6, Figure 6.6). Hayter *et al.* (1992) showed that glucose-limiting conditions during a perfusion culture resulted in an increase in nonglycosylated interferon-gamma. In Chapter 6, Figure 6.6, the glucose consumption for the suspension and Cytopore cultures are shown. Both the 37°C suspension and Cytopore cultures show the complete consumption of glucose at the end of the culture period. The glucose is completely consumed by day 5 in the suspension 37°C culture and day 6 in the Cytopore 37°C culture. For the temperature-shifted cultures, the glucose levels remain high even at the end of the cultures where between 1 to 1.5 g/L remains at the end of the culture. The low glucose uptake in the temperature-shifted culture could explain the higher percentage of glycosylated protein compared to the

nonglycosylated protein. However, this explanation is not sufficient for the suspension temperature-shifted culture.

Another explanation that can be used to describe the differences in site occupancy of the glycans can be the differences in pH. Borys *et al.* (1993) reported that the maximum glycan occupancy of a protein occurs at pH between 6.9 and 8.2. However, we cannot use this to explain our results, as the pH within the bioreactor was maintained at 7.1 throughout the culture period. However, NaOH was added to maintain the pH close to 7.1, and the although the pH was stabilized at 7.1 ± 0.1, the addition of higher volumes of NaOH was required to keep the pH constant.

In CHO cells the production of glycoproteins yields a mixture of bi-, tri-, tetra-antennary fully sialylated or lacking sialic acid and/or galactosylated glycans (Ashwell and Harford 1982; Cumming 1991; Diaz *et al.* 1989). This is due to different transferases that would be present within the cell line that would lead to the difference in terminal structures of the glycan. As well, there is a great dependence on the cell culture processes: the cell environment, process conditions, growth rate, and specific productivity (Weikert *et al.*, 1999).

The decreased sialylation in the Cytopore cultures may be attributed to the quick processing of the proteins and the glycans within the cell. The cell specific recombinant protein productivity in these cultures is high. Thus the rapid protein processing would decrease the relative time available for sialylation in the golgi. Another reason maybe the availability of sialic acid present in the culture, or alternatively the availability of the precursors; ManNAc and pyruvate. A decline in the dissolved oxygen has also been

shown to reduce sialyltransferase activity, specific productivity and sialic acid content (Chotigeat *et al.*, 1994).

The decreased sialylation that we showed in Cytopore microcarrier cultures compared to control suspension cultures is consistent with that found by others. Watson *et al.* (1994) showed reduced sialylation of N-glycans isolated from human tissue kallikrein produced from CHO cells on Cytodex microcarriers, compared to suspension cultures. Suspension cultures had more complex oligosaccharides, but the difference was much less greater than the sialylation. This finding was also consistent with that of Gawlitzek *et al.* (1995) who reported that the use of Cytodex and serum in cultures altered the glycosylation of a human interleukin-2 variant produced in BHK-21 cells, shifting the glycan profile to proximal fucosylated and unfucosylated biantennary and 2,4-branched triantennary structures. Also the sialylation rate was very low compared to cultures that were not supplemented with serum (20 %). The production of gp120 in HeLa cells showed consistent glycosylation in cultures using Cytodex 3 (Bleckwenn *et al.*, 2005).

No changes in glycosylation were reported with the production of EPO in CHO cells grown in weighted macroporous microcarriers (Cytoline) coupled with a fluidized bed bioreactor compared to suspension cultures (Wang *et al.*, 2002). However, glycans on gamma-interferon produced in CHO cells from a perfusion fluidized bed reactor culture had increased sialic acid content of tri- and tetra-antennary glycans after 210 h of culture (Goldman *et al.,* 1998). The N-linked glycan profile analyzed from an immunoglobulin (IgA) produced in a hollow fiber system showed a significant decrease in the amount of

sialylation compared to cultures in a continuous stirred reactor and a fluidized bed reactor (Schweikart *et al.*, 1999).

However, the effects of culture parameters can be varied when comparing the production of different recombinant proteins. Nam *et al.* (2007) report that there is an increase in the amount of sialic acid present on secreted alkaline phosphatase produced in CHO cells in the presence of Cytopore at lowered culture temperatures when compared to 37°C suspension cultures. The production of IFN-gamma at low temperature showed no effect on the glycosylation quality of the protein (Tan *et al.*, 2008). Trummer *et al.* (2006) show that inducing temperature shifts to CHO cells producing a heavily glycosylated hybrid protein Epo-Fc decreased sialylation.

Weikert *et al.* (1999) reported on the complete processing of terminal structures of TNK-tPA and TNFR-IgG oligosaccharides by engineering the cell lines to express high levels of human β1,4-galactosyltransferase and/or α2,3-sialyltransferase. The overexpression of these enzymes within the CHO cell lines led to a higher homogeneity of the processed glycans on TNK-tPA, and thus led to a longer mean residence time in rabbit models where the overexpression clearance rate was 2.0±0.3 mL/min/kg compared to 1.42±0.3 mL/min/kg. This experiment clearly shows that increased sialylation increases the efficacy of the recombinant protein.

Most N-glycans of recombinant glycoproteins studied are incompletely sialylated. In vivo, this may be due to (1) deficient Golgi α2,3 sialyltransferase activity (Weikert *et al.*, 1999), (2) high rate of transport of the glycoprotein substrate through the golgi (Nabi and

Dennis, 1998), (3) competition between glycoprotein substrates for sialylases in the golgi (Kornfeld *et al.*, 1964), (4) steric inaccessibility of glycan acceptor (Weikert *et al.*, 1999), and/or (5) control of nucleotide-sugar substrate (CMP-NeuAc) transport into the Golgi lumen.

The sialylation within the culture can also be decreased via degradation by sialidase enzymes. However, this is less likely to be the case in our cultures, due to the maintenance of high culture viability throughout the culture (Sliwkowski *et al.*, 1992). The release of sialidase, a cytosolic enzyme released into cell culture upon cell lysis, has been found to be the cause of post secretory degradation (Gramer and Goochee, 1993; Wond *et al.*, 2005).

The nutrient supply is an important factor on the sialylation of the recombinant protein. Lower levels of glucose (<0.70 mM) or glutamine (<0.1 mM) have been shown to reduce sialylation in the production of IFN-gamma (Wong *et al.*, 2005). In our case, the glutamine present in the culture was greater than 0.5 mM throughout the culture period. In the case of the temperature-shifted cultures (suspension and Cytopore) the glutamine concentration present was 1.5 mM at the end of the culture. Thus the cultures were not glutamine deficient (Chapter 6, Figure 6.7).

It was hypothesized that a reduction in specific productivity would increase the site occupancy of glycans on a recombinant glycoprotein, and thus it was predicted that lower temperature cultures would increase the site occupancy of cultures. However Andersen *et al.* (2000) showed that there is a lack of correlation between specific productivity and site occupancy. Thus they argued that the site occupancy is dependent on the growth phase of

the cells, and cells in G0/G1 would produce glycoproteins with higher site occupancy. Goldman *et al.* (1998) showed a higher cell specific productivity of IFN-gamma in perfusion cultures (397 IU/10^6 cells/h) compared to stirred cultures (303 IU/10^6 cells/h), and correlated the increase in site occupancy to the higher specific productivity. However, they reported similar sialic acid content in both stirred tank reactors and perfusion, where sialic acid content was found to be 1.93 to 1.97 SA/N-glycan and 1.91SA/N-glycan, respectively. Kunkel *et al.* (1998) reported a correlation between an increase in dissolved oxygen and sialic acid in of the production of IgG.

The sialylation of the IFN-beta is important for the efficacy of the protein when used in treatment. Asialoglycoprotein receptors present in the liver and macrophages cause the removal of glycoproteins from the circulatory system, and thus decreases the half-life of the protein in the circulatory system (Weiss and Ashwell, 1989). The sialylation was shown to decrease in both the 37°C and temperature-shifted Cytopore cultures when compared both suspension cultures. The decrease is especially prominent in the temperature-shifted Cytopore culture where 18 % of the glycoforms were of the A2G2FS0 moiety; however there is a shift to less sialylated forms in the 37°C Cytopore culture. The reason for this may be due to the increase in protein production within the 37°C Cytopore culture, as the specific productivity of the cells is 1.4510 units/cell-day compared to the 37°C suspension culture at 0.6045 units/cell-day. The temperature-shifted suspension culture had a Qp of 0.7710 units/cell-day, which was relatively similar to the temperature-shifted Cytopore culture with Qp of 0.8843 units/cell-day. Although the Qp of both temperature-shifted cultures were similar, the overall protein yield in the temperature-shifted Cytopore culture was much greater, at 5-fold higher than the 37°C

suspension culture. There was also a higher level of heavier glycosylated proteins present in the temperature-shifted Cytopore culture, where close to 15 % of glycoforms had a GU of greater than 8.3.

Section A - Discussion

The ultimate goal of using microcarriers in cell culture was to achieve an increase in the volumetric and specific productivity, with analysis into the product quality of the recombinant protein produced.

Initially, I compared microporous (Cytodex) and macroporous (Cytopore) microcarriers for the growth and productivity of recombinant IFN-beta and tPA produced by CHO cells. The use of Cytodex did not significantly enhance recombinant protein production, where IFN-beta producing cells produced a lower titre in higher concentrations of Cytodex and tPA production was slightly enhanced (up to 1.6-fold) in the presence of Cytodex. The recombinant protein production was greatly enhanced in the presence of Cytopore where there was an increase of up to 2.5-fold of both IFN-beta and tPA compared to suspension cultures. The specific productivity was also enhanced by up to 2.5-fold in the case of Cytopore 1 and up to 5-fold in the case of Cytopore 2.

The enhancement was further amplified when IFN-beta cells growing on Cytopore 2 microcarriers were temperature-shifted to 32°C after 2 days at 37°C. The temperature change increased protein yields by over 5-fold compared to a suspension at 37°C. Furthermore the temperature shift and Cytopore increased the overall glycosylation of the protein by 15 % compared to the suspension culture. Also, the temperature shift and Cytopore led to lower sialylation when comparing the A2G2FS0, A2G2FS1 and A2G2FS2 glycoforms, where the suspension cultures had a degree of sialylation of 82.3 % (37°C) and 79.2 % (37 to 32°C), the Cytopore cultures showed a degree of sialylation

of 72.5 %, and both the Cytopore and temperature shift led to a degree of sialylation of 65.5 %.

The enhancement of protein titres in the microcarrier cultures was attributed to the high cell density attained (greater than 10^8 cells/cm^3) within the macroporous microcarrier cultures. The cells, once inoculated, enter within the pores and grow to high cell densities. The cells within the Cytopore microcarriers may be in a high productivity state, where autocrine factors could encourage high recombinant protein productivity (Yamaguchi *et al.*, 1997). This high cell density state may not be supported by the Cytodex microcarriers, where in most cases the cells grow in monolayers on the microcarrier bead. However, this does not explain the case for the tPA producing cell line, where the higher Cytodex microcarrier concentration (5.0 mg/mL) had higher volumetric yield of tPA (1.6-fold) compared to the suspension culture.

The benefits of using porous microcarriers in cultures are that the concentrations of porous carriers used in cultures could be much lower than solid carriers, the growth of cells within the pores would allow for protection from damage from shear stresses, can accommodate for anchorage dependent and anchorage independent cell lines, and there are fewer cells suspended in supernatants (Xiao *et al.* 1998). These reasons could be used to explain the high cell densities attained within the Cytopore microcarriers.

The increased production of recombinant protein from high cell density cultures is an important method to maximize protein production from cultures. In particular, the use of Cytopore in scaled-up processes can maximize recombinant protein production up to 5-fold compared to suspension cultures. The increased production of recombinant proteins

from entrapped cells has been alluded to by many researchers; however, in this study we were able to show a definite correlation between an increase in cell density within the microcarrier and the specific productivity of cells.

The use of microcarriers in the small scale spinner cultures enabled us to study the trend between specific productivity and increased cell entrapment. By scaling these cultures up by 20-fold, in a 2L bioreactor culture, we were able to increase volumetric production by 2.25-fold. From the experiments done in this chapter, I would recommend the use of Cytopore 2 microcarriers in large scale production of these recombinant proteins. For the production of proteins prone to aggregation, the use of a temperature-shift regime, could enhance the production further, as was the case for the production of IFN-beta, where a temperature-shift from 37°C to 32°C, in the presence of Cytopore 2 yielded over 2-fold greater protein than Cytopore 2 alone. However, this maximized production is offset by a decrease in sialylation.

Section B – The Effects of PF68 on Cell Growth, Recombinant Protein Production and Glycosylation

Introduction

PF68 is a non-ionic surfactant used in cell culture to protect cells from high shear forces that cells are exposed to in suspension cultures. The trend in media development is to move from serum-based media to serum-free media. This ensures the production of recombinant proteins for human therapeutics devoid of animal components. Some disadvantages of using serum in media formulations include: batch to batch variations; the addition of chemically undefined materials; inconsistent growth/productivity; serum proteins compromising the extraction and purification procedures for cell secreted proteins; the possibility for virus and prion diseases. The removal of serum in culture leads to the removal of shear protective elements from the media, such as albumin and bulk proteins (Keenan *et al.*, 2006), and thus cells are more inclined to be affected by shear forces, and thus the importance of adding detergents to the media.

There are two main theories as to the effects of PF68 on cells in culture a) mechanical effect where PF68 coats bubbles and affects the cell to bubble interaction and b) a biological effect whereby PF68 interacts with the plasma membrane. Many studies have studied the mechanical effect describing the stabilization of the foam layer by PF68 (Handa-Corrigan *et al.*, 1989) and the reduction of the cell to bubble interactions in the presence of PF68 (Chattopadhyay *et al.*, 1995; Meier *et al.*, 1999; Michaels *et al.*, 1995). The incorporation of PF68 by the plasma membrane has also been described by Palomares *et al.* (2000) who describe the long term effects of PF68 after its removal from culture. The uptake of PF68 by cells has also been described by Gigout *et al.* (2008) who

describes PF68 being internalized and accumulated in the endocytic pathway. Thus the debate of the mechanical and biological effects of PF68 on cells is ongoing.

In this section, I investigate the effects of PF68 on static cultures, the effects of PF68 on shear damage and proteolytic damage of cells, and the effects of PF68 on recombinant protein production and glycosylation.

Chapter 8

8. PF68 Induces Adherence of cells*

8.1. Introduction

Cell adherence is induced by the interaction of the cell membrane and the growth surface. The interaction between these two surfaces and Van der Waal's or electrostatic attraction causes the cell adherence (Butler 2004). There are three different classifications of junctions between cells: occluding junctions, communicating junctions and anchoring junctions (Schneeberger and Lynch 2004). Junctions that seal cells together, and prevent small molecules from leaking from one side to another are defined as occluding junctions. Communicating junctions prevent the passage of chemical or electrical signals from one cell to another. Anchoring junctions are responsible for the attachment of cells to either their neighboring cells or to extracellular matrices.

In culture, serum fibronectin, as well as that produced by the cells, can bind to both the collagen substrates and the tissue culture plastic surface and mediate the attachment of cells (Kleinman *et al.* 1981). All vertebrate cells have an unevenly distributed negative surface charge and thus are attracted to positively-charged surfaces. Due to the propylene oxide blocks on either arm of PF68, which are hydrophobic, PF68 molecules are able to attach to the cell and thus extend their hydrophilic tails (polyethylene oxide) out into the media. This would decrease the attachment of cells to surfaces.

Cell adherence consists of initial contact of the cell with the surface, cell spreading on

* The contents of this chapter have been included in a paper: Tharmalingam, T., Ghebeh, H., Weurz, T., and Butler, M., 2008. **Pluronic enhances the robustness and reduces the cell attachment of mammalian cells.** Molecular Biotechnology. 39(2):167-177. Work reported in the paper completed by others was omitted from this chapter.

the surface, and then cell differentiation and growth. Attachment can be divided into four different phases: initial attachment to the surface; spreading by spheroidal cells where cells are more adherent due to increased contact area and bond density; flattened cells but with less adhesiveness than phase two cells; and fully attached and extremely flat cells (GE-Healthcare 2005).

Cell binding to a surface requires the secretion of an extracellular matrix (ECM) molecules that adsorb onto a surface. The attachment process involves transmembrane adhesion receptors (such as cadherins, integrins, and N-CAMs) and adsorbed adhesion proteins (such as fibronectin, vitronectin, and laminin) (Chothia and Jones 1997). The attachment of cells to a surface, often leads to cell speading. This speading is due to complexes of protein (such as microtubules, actin filaments, and ligand-bound adhesion receptors) that assemble at adhesion sites, also known as focal contacts (Bereiter-Hahn *et al.* 1990; Burridge and Chrzanowska-Wodnicka 1996). These protein complexes form around transmembrane proteins such as integrins, which bind ECM molecules and actin filament bundles within the cytoskeleton (Burridge and Chrzanowska-Wodnicka 1996). As a result, the cells often spread out on the surface to which they are adherent, thus giving a distinct morphology to cultured cells.

In this section, we describe the effects of different PF68 concentrations on the growth of cells in stationary T-flask cultures. The removal of PF68 from T-flask cultures induced cells to grow attached to the T-flask surface, and thus studies were done to investigate this property further.

8.2. Results

8.2.1. CHO cell attachment to T-flasks with the removal of PF68

In the absence of PF68, CHO cell counts appeared to decrease over time. Initially this was believed to be due to an inhibitory effect when cells were removed from PF68. However, upon further examination, there appeared to be an increase in the number of cells attached to the T-flask and a corresponding decrease in the number of cells in suspension. The cells were visualized by light microscopy and are shown in Figure 8.1. The left panel (A) shows CHO cells grown in suspension in the presence of BioGro CHO SFM which contains 0.1 % PF68. The cells are spherical and do not show their natural fibroblastic cell appearance. The right panel (B) shows cells that were grown in the absence of PF68. The cells are found to adhere and take on fibroblastic growth patterns similar to their state in serum containing media.

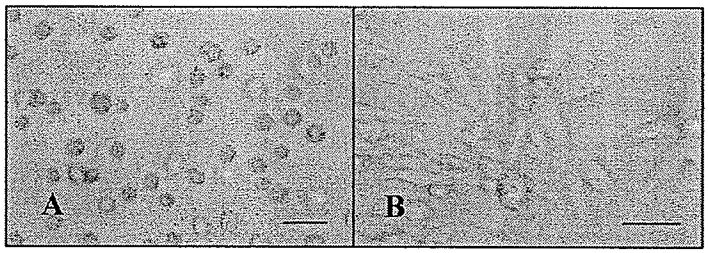


Figure 8.1: Light microscopy photographs showing CHO cells found to grow in suspension in the presence of 0.1 % PF68 (A) and those found to grow adherent in the absence of PF68 (B). The bars indicate 50 μm.

Initial studies showed that there was a progressive reduction of cells in suspension in T-flask cultures when the PF68 concentration was decreased from a concentration of 0.1 % (w/v) to 0 %. In parallel there was a progressive increase of cells that were found to adhere to the surface of the T-flasks. The total number of cells within the T-flask was found to be relatively equivalent over the concentration range of PF68.

This trend was confirmed when growing cells in PF68-free and in the presence of PF68 (0.1 %). The percent adherence was calculated by taking the adherent cell density as a function of the total cell population.

The cells for the purpose of adherence studies were inoculated at a cell concentration of $1x10^5$ cells/mL. The cells were grown in the absence of PF68 for 3 passages prior to being exposed to PF68, to ensure no residual carry-over effect of PF68 between cultures from the cells or the media. This was minimized by washing cells in PBS-EDTA and fresh PF68-free media prior to inoculation.

Figure 8.2 shows the cell growth patterns of representative T-flask cultures. Adherent and suspension cell concentrations were enumerated separately then compared. The combined concentrations of adherent cells and suspension cells gave the total cell concentrations within the T-flask culture. The total cell concentrations were relatively constant under all concentrations of PF68. The total cell concentrations increased from $3.48x10^6$ cells/mL in PF68-free media, to a maximum of $4.85x10^6$ cells/mL in 0.05 % PF68, and then slowly declined to $3.13x10^6$ cells/mL in 0.1 % PF68. The suspended cells followed a similar pattern; however under PF68-free conditions the cells in suspension were minimal compared to the suspension cell concentrations in 0.03 % PF68 and higher. There appeared to be an exponential increase of cells growing in suspension from 0 % to 0.03 %, at which there appeared to a plateau, with a slight decrease towards the higher concentrations approaching 0.1 %. At lower concentrations of PF68 the suspended cell concentrations were compensated for by taking into account the attached cell concentrations. This was due to there being more adherent cells at lower concentrations

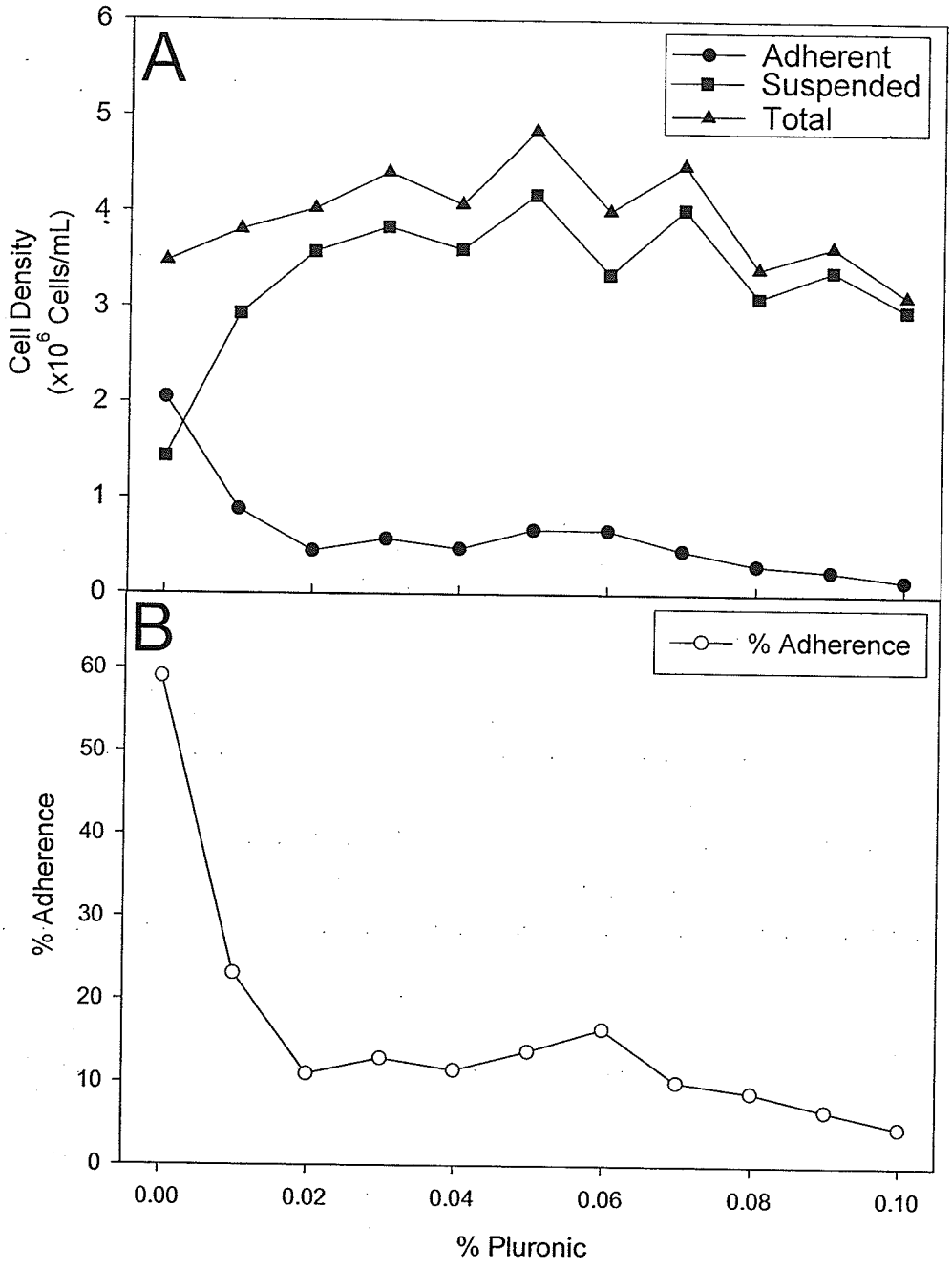


Figure 8.2: The effect of Pluronic on IFN-beta CHO cell adhesion. The proportion of cells that are adherent to the T-flask, grown in suspension and the total cell counts (A). The cells were then inoculated into T-75 (150 cm^2) for 4 days. The suspended cells were washed in PBS and counted independently. The adherent cells were removed by the addition of trypsin, then washed in PBS and counted independently. The cells were enumerated by the trypan blue exclusion method and percent adherence (B) was calculated by comparing the adherent cells to total cell population.

of PF68, where there appeared to an exponential decay of adherent cells as the concentration of PF68 was increased. The adherent cell concentrations were decreased from $2.05x10^6$ cells/mL, in PF68-free media to $0.15x10^6$ cells/mL in 0.1 % PF68.

The cell adherence decreased from 59 % to 4.7 % as the PF68 concentration increased from the 0 to 0.1 % PF68 (Figure 8.2B). Visualizing the cells through the light microscope (Figure 8.1B) showed that the attached cells in the absence of PF68 were fibroblastic and elongated. However cells attached in higher concentrations of PF68 were found to be more spherical.

8.2.2. Adherence of CHO cells with different PF68 concentrations

The concentration of PF68 in Biogro CHO-SFM was 0.1 % PF68. And at this concentration it is apparent that less than 5 % of cells are adherent to the T-flask surface. However, decreasing this concentration increased the proportion of cells that grew adherent to the T-flask surface, up to the complete absence of PF68 where 70 % of cells could be adherent. Figure 8.3A and 8.3B show the increase of adherent cells in IFN-beta producing CHO cells and tPA producing CHO cells, respectively.

8.2.3. The long term effects of PF68 on cell adherence

We then tested whether the adherent cells could be returned to PF68, and whether the re-exposure to PF68 would allow the cells to return back to their "natural" state of spherical morphology. The cells were grown in PF68 media, during continuous passaging. The first passage out of PF68 is identified as the first passage of the experiment in Figure 8.4. The percentage of adherent cells in this first passage was 5 %.

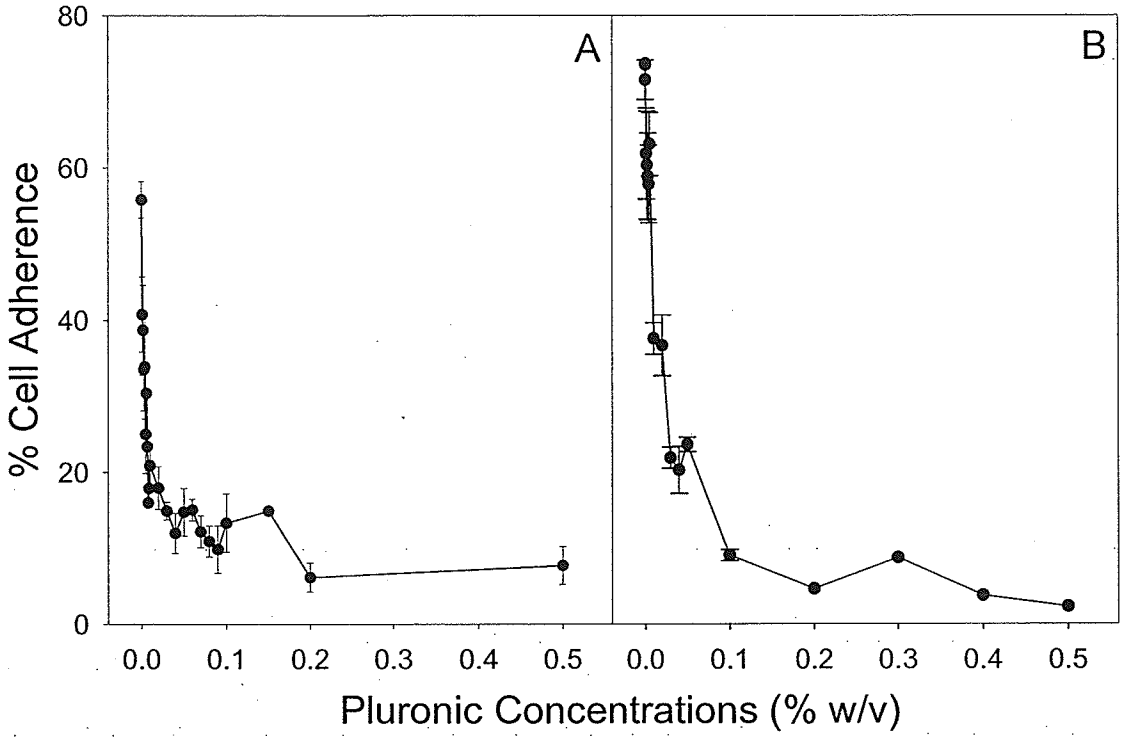


Figure 8.3: Cell attachment to T-flask surface as the PF68 concentration is decreased. The IFN-beta producing (A) and tPA producing (B) CHO cells were grown in 0 % PF68 for 3 passages prior to the experiment to ensure the complete removal of PF68 from the cells and the media. The cells were then inoculated into T-75 (150 cm^2) for 4 days. The suspended cells were washed in PBS and counted independently. The adherent cells were removed by the addition of trypsin, then washed in PBS and counted independently. The cells were enumerated by the trypan blue exclusion method and percent adherence was calculated by comparing the adherent cells to total cell population.

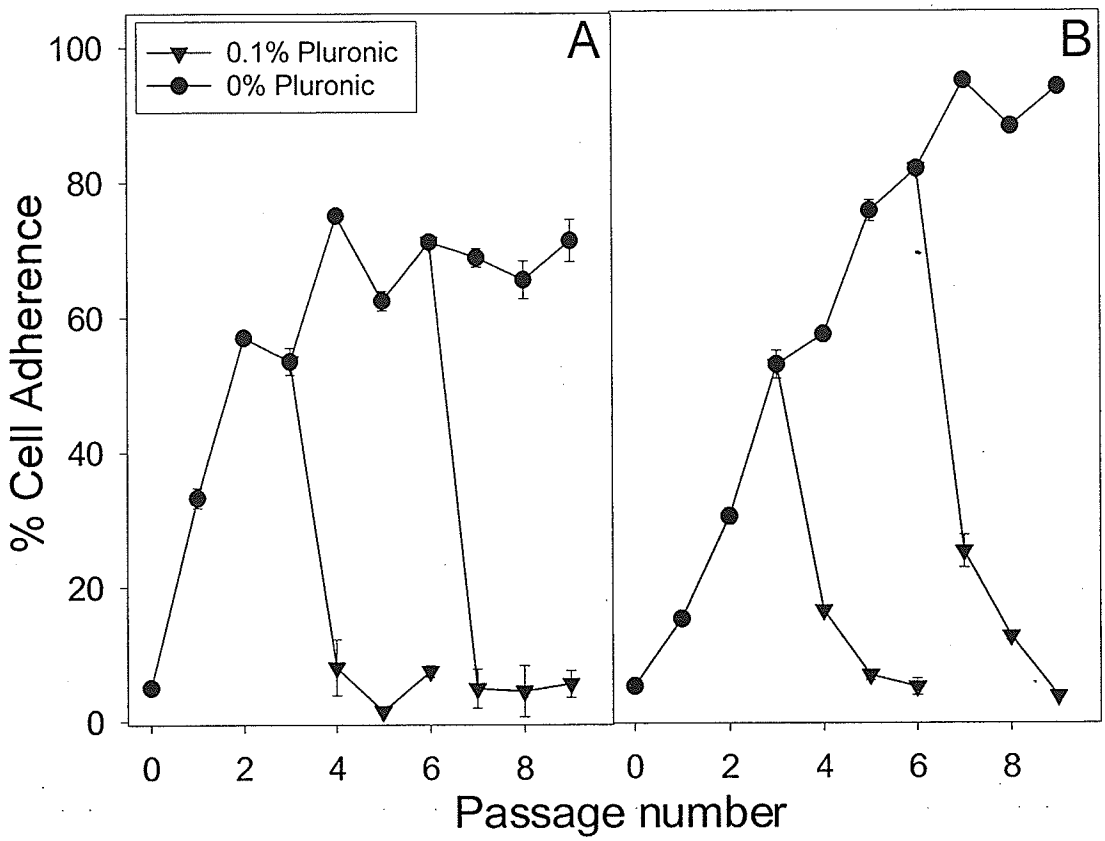


Figure 8.4: The removal of PF68 over multiple passages. IFN-beta producing (A) and tPA producing (B) cells were grown in PF68 containing medium prior to the experiment. At the first passage the cells were removed from PF68 into T-75 T-flasks (150 cm^2). At passage 3 and 6 the cells were split into PF68 containing medium (▼) or PF68-free medium (●). The cells were enumerated by the trypan blue exclusion method where suspended cells and adherent cells were removed and enumerated independently. The percent cell adherence was calculated by comparing adherent cells to total cell population.

Over subsequent passages in PF68-free medium there appeared to be an increase in cell to surface adherence. The percentage adherence of IFN-beta producing cells at passage 3 was 55 %. Over the next passage the culture was split, such that one group was followed to grow in PF68 and the other in PF68-free media for 3 passages. The cells were then returned to the media containing PF68, at which point cell adhesion decreased to 5 %. The cells grown in PF68-free media continued to grow adherent in all three passages from 55 % initially, to 65 %. Cells growing in media containing PF68 continued to grow in suspension for 3 passages, with adherent cells taking about 5 % of the population of cells.

The experiment was carried over a total of 9 PF68-free passages. The adherent IFN-beta cells increased to 70 % where tPA producing cell line increased to 95 % adherence. At passage 6, the cells were split into 2 groups, one was incubated in the presence of PF68, and the other remained in PF68-free media. The cells were found, once again, to return to spherical suspension cells once PF68 was reintroduced in the media. However, cells that remained in PF68-free media continued to grow in a fibroblastic cell appearance. There were however, differences between the 2 different cell lines. IFN-beta producing cells were less adherent than the tPA producing cell line in the absence of PF68.

8.3. Discussion

The results clearly indicate that as the concentrations of PF68 increased in stationary T-flask cultures, cell adherence decreased. This trend can be explained by the coating of the detergent to the surface of T-flasks, and thus causing the hydrophobic tails to remain

exposed on the surface of T-flasks. As cells are added to the flasks, the hydrophobicity of the detergent deters any adherence that would usually be mediated by this cell to surface attraction.

Cell adherence was visible in Figure 8.1, where the cells were found to spherical in the presence of PF68. The absence of PF68 from culture media induced cells to grow attached to the T-flask surface. However, there was spherical cells found in the cell samples, probably due to the attachment phase (GE-Healthcare 2005). When cells are inoculated into the T-flask the cells appear to be spheroidal; however within days most cells appear to adhere nonetheless there are cells that remain in suspension even in the end of the passage.

The decreased hydrophobicity of the outer membrane brought about by PF68 (Ghebeh *et al.*, 2002) may explain the reduced adherence of the cells in stationary culture by a reduced interaction of the cells with the tissue culture-treated attachment surface. It was clearly shown that a PF68 concentration at 0.1 % can reduce the proportion of attached cells from 70 % to minimal levels. This also reduces cell-cell interactions and as a consequence fewer cellular aggregates were observed in PF68-supplemented cultures Previous reports by Keen & Rapson (1995) and Bentley *et al.* (1989) have shown enhanced growth in the presence of PF68 in stationary cultures even in the apparent absence of shear forces. This is likely to be due to the higher growth rate often found in cultures of suspension cells compared to surface-attached cells. There may also be an interaction of PF68 with the flask surface to increase the wettability of the culture substratum (Detrait *et al.*, 1999). Alternatively, but less likely, explanations offered for

the effect of PF68 in stationary cultures are an increase in the nutrient uptake (Mizrahi, 1975) or an increase in growth by the impurities in commercial batches of PF68 (Chisti, 1999).

The adherence effect was found to be reversible, as PF68 was removed the percentage of adherent cells increased. However, once PF68 was reintroduced into the stationary T-flask cultures, the percentage of adherence decreased. Once visualized through the microscope, the cells appeared to have mixed attachment patterns. Some cells were found to be flat and stretched, as characteristic of fibroid cells, whereas other cells attached but remained spheroidal.

The adherence of cells was rapidly reversible upon treatment with PF68-free media as shown over multiple culture passages. This indicates that PF68 is easily removed from the cell surface and suggests that it is not incorporated into the membrane, so that its effects are not prolonged as might be the case for constituent membrane components such as fatty acids (Butler *et al.*, 1999). This is confirmed by the report of Michaels *et al.* (1991) who showed that long-term exposure to serum makes cells more shear resistant whereas there is no benefit of long-term exposure to PF68. The reversible effect of PF68 on cell adherence is clearly an advantage in controlling the state of either stationary or agitated cultures.

Dewez *et al.* (1996) reported that the presence of PF68 on bacteriological grade polystyrene prevented the cell adhesion of Hep G2 human epithelial cells (Dewez *et al.*, 1996). The inhibited attachment of the PF68 on the polystyrene was reported to be due to the increase in hydrophobicity of the substratum. Two years later the same group showed

that this inhibition of cell to surface adherence when PF68 was present was evident with three other cell lines: MSC 80 mouse schwannoma, PC 12 rat adrenal pheochromocytoma, and rat hepatocytes (Dewez *et al.*, 1998). Furthermore, cell adhesion was not influenced by PF68 when the substratum was pre-conditioned with ECM proteins, such as collagen, laminin, and fibronectin (Dewez *et al.*, 1999).

Various pluronics have been studied for their impact on cell adherence. The results are comparable to that found by Biran *et al.* (1999), where cell adhesion of astrocytes were decreased with the increased hydrophobicity of the growth surface where the surfactant used in this study was PF108 (PEO_{132}-PPO_{56}-PEO_{132})(Biran *et al.* 1999). PF108 was also shown to inhibit NIH 3T3-J2 murine fibroblast cell adhesion on glass for up to 4 weeks (Liu *et al.*, 2002). Another study of cell to surface adsorption used PF127 (PEO_{98}-PPO_{67}-PEO_{98}) to show the ability to control CHO cell adherence on liposomes composed of different lipid sources (egg phophatidylcholine, dioleoloxytrimethylammonium propane methyl sulfate, dipalmitoyl rhodamine phosphatidylethanolamine, and distearoyl phosphatidylethanolamine) by varying concentrations of PF127 (Chandaroy *et al.*, 2002).

The effect of Pluronics on the inhibition of cell to surface adherence is not specific for mammalian cells. Levy *et al.* (2004) found that bacterial attachment to silicone surfaces in the presence of Pluronic P188 (PP188) was decreased to 3.02 % compared to a control with 22.2 %.

In this chapter, we reported on the effects of PF68 on cell adherence of two independent CHO cell lines. In the absence of PF68, cells grew attached to the surface of

T-flasks, where adding PF68 induced cells to grow in suspension, in a concentration dependent manner. The effects of PF68 appeared to be reversible, as adding PF68 to cells that grew attached for numerous passages induced cells to grow in suspension. This work is important for media development, as the concentration of PF68 could influence the growth of cells.

Chapter 9

9. Use of PF68 for protection of cells in high shear conditions*

9.1. Introduction

Mammalian cell culture processes are used for the production of an increasing number of commercially important biopharmaceuticals that include vaccines, recombinant proteins, and monoclonal antibodies (Butler, 2005). One important criterion for scaling these processes is the use of serum-free media with minimal protein content and the absence of animal-derived components, as demanded by regulatory authorities in recognition of the dangers of animal-borne contaminants (Schroder *et al.*, 2004). However, the low protein content of the latest generation of media formulations may increase the risk of cell damage through high levels of shear stress introduced by sparging and stirring in the culture vessel (Van der Pol and Tramper, 1998). The shear sensitivity of mammalian cells to such an environment is variable and may depend on a number of factors including cell type, thickness of the cell membrane, cell size, growth rate, growth medium and the type and concentration of shear protective agents (Chisti, 1999).

In large-scale stirred-tank bioreactors of animal cell cultures (>2 L), transfer of oxygen via the air-liquid interface is not sufficient to supply the metabolic requirements of growth beyond 10^6 cells mL^{-1} and therefore gas sparging may be necessary (Butler, 2004). However, sparging generates bubbles that burst at the surface leading to detrimental effects on cells (Bavarian *et al.*, 1991; Chalmers and Bavarian, 1991; Jordan,

* The contents of this chapter have been included in a paper: Tharmalingam, T., Ghebeh, H., Weurz, T., and Butler, M., 2008. **Pluronic enhances the robustness and reduces the cell attachment of mammalian cells.** Molecular Biotechnology. 39(2):167-177. Work reported in the paper completed by others was omitted from this chapter.

1994; Kunas and Papoutsakis, 1990; Tan *et al.*, 1994; Trinh, 1994; Wen and Tan, 1999). This is caused by the retracting rims of bursting bubbles at the liquid surface that generate a jet with a speed of 3 m s^{-1} resulting in a mechanical shear force sufficient to lyse cells in the surrounding area (Cherry and Hulle, 1992). Agitation by impellers to mix a suspension cell culture or cell-attached microcarriers also generates shear forces. Nevertheless, in suspension cultures, with rates of impeller agitation below 800 rpm the shear forces generated from mixing do not damage cells where a bubble-free aeration device is deployed and the air-liquid interface is eliminated (Kunas and Papoutsakis, 1990). Otherwise in the presence of an air-liquid interface, agitation speeds above 200 rpm cause cell damage if a vortex is generated leading to air entrainment and therefore bubble bursting at the liquid surface (Kunas and Papoutsakis, 1990).

The protective effect of PF68 has been shown to occur by decreasing bubble-cell attachment (Jordan *et al.*, 1994; Michaels *et al.*, 1995), decreasing surface tension and thereby decreasing the energy generated from bubble bursting (Dey *et al.*, 1997; Ma *et al.*, 2004). PF68 may also increase cell membrane strength. Zhang *et al.* (1992) showed by micro-manipulation techniques that the critical tension to burst cells increased significantly in the presence of PF68. This suggests the possibility of incorporation or adsorption into the cell membrane (Goldblum *et al.*, 1990; Murhammer and Goochee, 1990).

The objective of this study was to investigate the multiple effects of PF68 on cell protection, repair and surface adhesion in transfected CHO cell lines in an attempt to better understand the mechanisms involved. The experiments were based on two CHO

cell lines that were transfected for heterologous protein expression. High shear rates were applied by increasing the agitation rate of the spinner flasks and by a cone-and-plate viscometer that offered a bubble-free environment for the cells.

9.2. Results

9.2.1. Agitated cultures

9.2.1.1. Spinner flasks

The removal of PF68 from spinner cultures appears to affect the cell stability after multiple passages in the absence of PF68. CHO cells producing tPA that were removed from PF68 over multiple (1 to 4) passages were inoculated in 100 mL spinner cultures and measured for cell density and cell viability. The cells were inoculated into spinner flasks with (Figure 9.1B) and without (Figure 9.1A) PF68, to test the shear sensitivity of the cells growing at a low stir rate (45 rpm). The PF68-free cells inoculated into media with PF68 and appeared to induce a similar growth pattern in each passage, where the cells enter exponential phase at day 2 reaching a maximum cell density of $2.5x10^6$ cells/mL by day 5 and slowly decline towards the end of the culture. Cells inoculated into PF68-free media appeared to show different growth characteristics, where cells from the first three passages out of PF68 reached a maximum of $1.5x10^6$ to $1.75x10^6$ cells/mL by day 5 or 6 and slowly declined towards the end of the culture. Cells from passage 4 out of PF68 appeared to be affected by the shear forces within the spinner, as the cells reached a maximum of $0.5x10^6$ cells/mL by day 5.

Measurement by trypan blue indicated that cell viability of all cultures remained above 85 % for all spinners until day 5, except for the cells removed from PF68 at

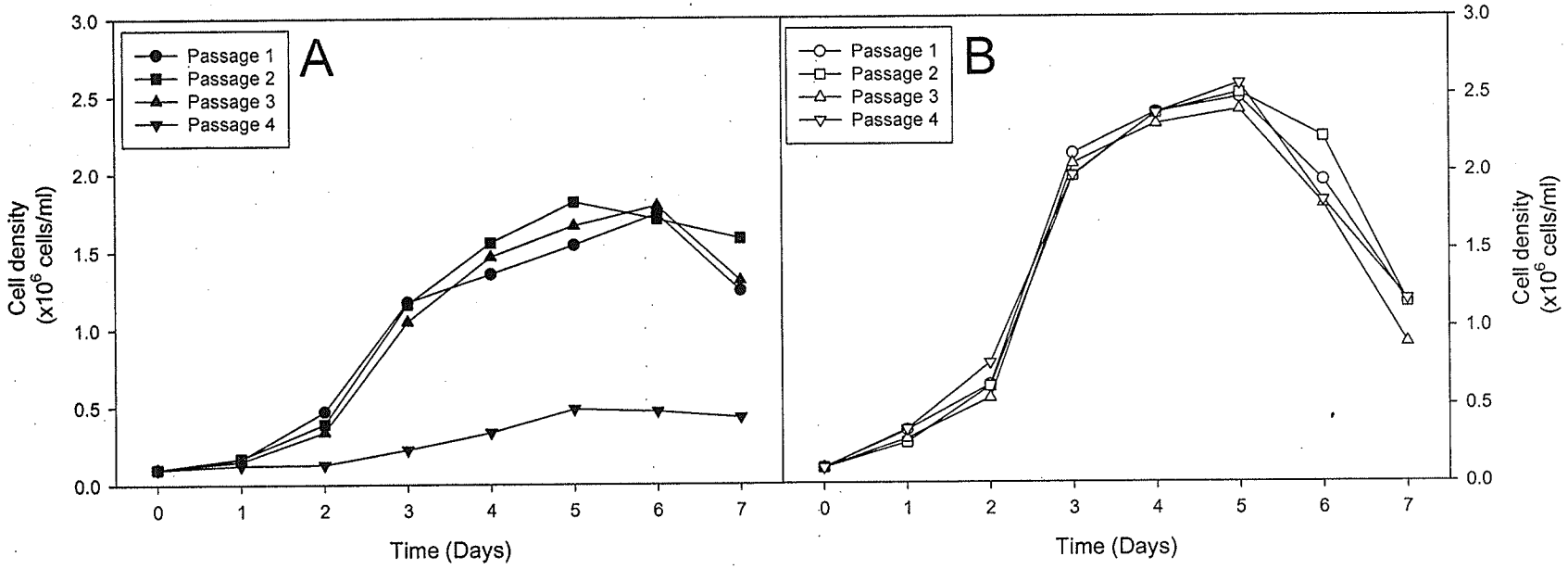


Figure 9.1: Representative cell growth patterns of tPA producing CHO cell lines over multiple passages in the absence of PF68. After each passage in the PF68-free media, the cells were grown in spinner flasks stirred at 45 rpm containing (A) PF68-free or (B) PF68 containing media (0.1 % w/v). Cell densities were estimated using the trypan blue exclusion method.

passage 4, growing in PF68-free media. The viability for this culture decreased to 80 % by day 1 of the culture, and remained at roughly 80 % until day 5, after which it declined to 50 % at day 7.

The IFN-beta producing cells were removed from PF68 for 3 passages and then reintroduced into PF68 or continued a fourth passage in PF68-free media. The growth of cells under both conditions is shown in Figure 9.2. The cells under both conditions grew to similar cell concentrations when inoculated into spinners containing media with and without PF68. Both spinner flasks were inoculated at $1x10^5$ cells/mL and counted daily by haemocytometer using the trypan blue exclusion method. The cells both in the presence and absence of PF68 entered an exponential phase at day 2, and continued growth until day 6, with a maximum cell density of $4.20x10^6$ cells/mL, after which the viable cell population declined to $3.60x10^6$ cells/mL. The IFN-beta cells were monitored for growth in further passages of PF68-free medium, and it seemed that the cells continued similar growth curves (data not shown), thus showing that the cells are unaffected by the shear stresses induced at 45 rpm in the absence of PF68.

The removal of PF68 for 3 passages ensured that the possibility of cell carryover of PF68 from passage to passage was eliminated. The differences in the growth of the tPA producing (Figure 9.1) and the IFN-beta producing (Figure 9.2) CHO cell lines in the absence of PF68 shows a difference in the cell resistance to shear.

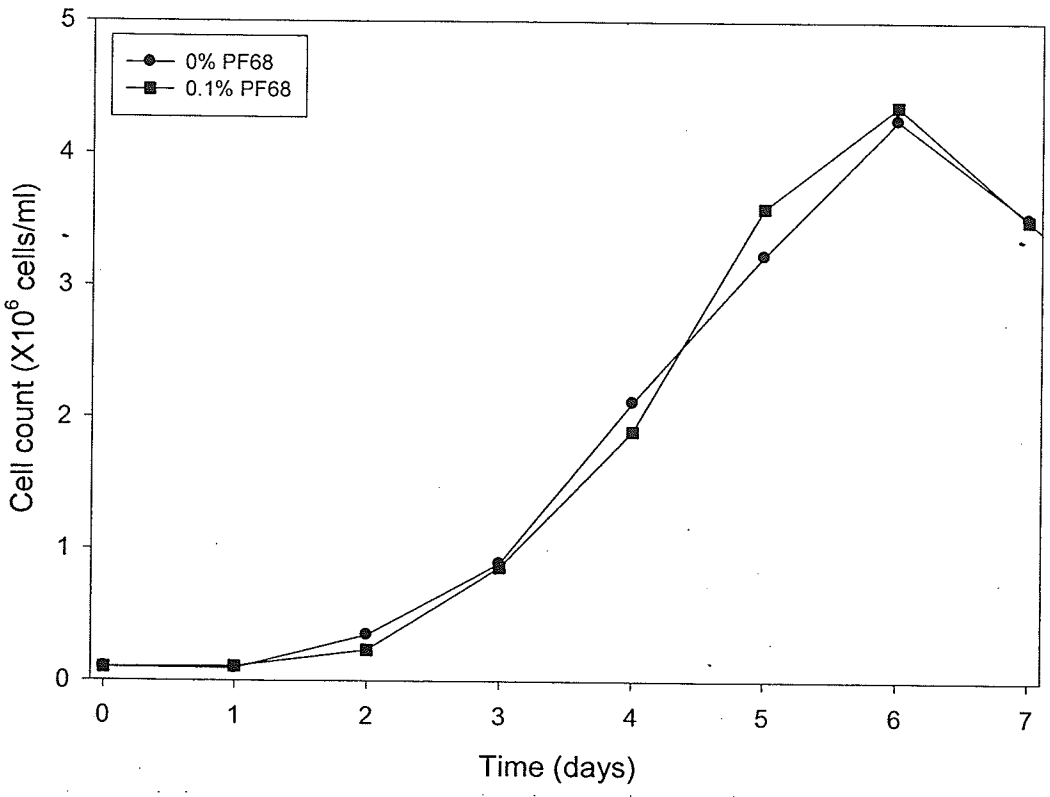


Figure 9.2: The growth of IFN-beta producing CHO cells after the removal of PF68 after 3 passages. The cells were removed from PF68 for 3 passages before being inoculated into the absence (●) or presence (■) of PF68. Viable cell concentration was estimated using the trypan blue exclusion method (0.5 % w/v).

9.2.1.2. Bioreactor

The effect of PF68 in agitated cultures was determined in a controlled stirred-tank bioreactor with a stirring speed of 100 rpm (Figure 9.3). The IFN-beta producing cells were grown in PF68-free medium for 3 passages prior to inoculation into the bioreactor culture (2 L) at a seeding density of $1x10^5$ cells/mL. Cells in PF68 (0.1 %) containing medium grew at a maximum specific rate of 0.018 h^{-1} and reached a maximum cell density of $4.3x10^6$ cells/mL after 7 days of culture. However the cells in PF68-free medium showed poor growth with a maximum cell density of only $0.45x10^6$ cells/mL after day 4. The significant difference in growth shows the protective effect of PF68 in the agitated environment of the bioreactor ($p<0.01$).

9.2.2. Cell damage at high shear rates

Shear stress can be induced in cell culture by high stirring rates or gas sparging in culture. In order to quantify these protective effects of PF68, we developed two systems to measure rates of cell damage. Cells were exposed to a high agitation rate in a spinner flask. In this system at agitation rates above 250 rpm, a liquid vortex developed in the culture causing gas entrainment and associated damage to cells. We chose a standard stirring rate of 500 rpm that would allow measurement of loss of cell viability over a reasonable period of time (Butler *et al.*, 1999). In a second system a cone-and-plate viscometer was used to generate high shear stress in a bubble free environment. This would allow rates of cell damage to be determined in an environment of liquid shear but without the effects of a gas-liquid interface.

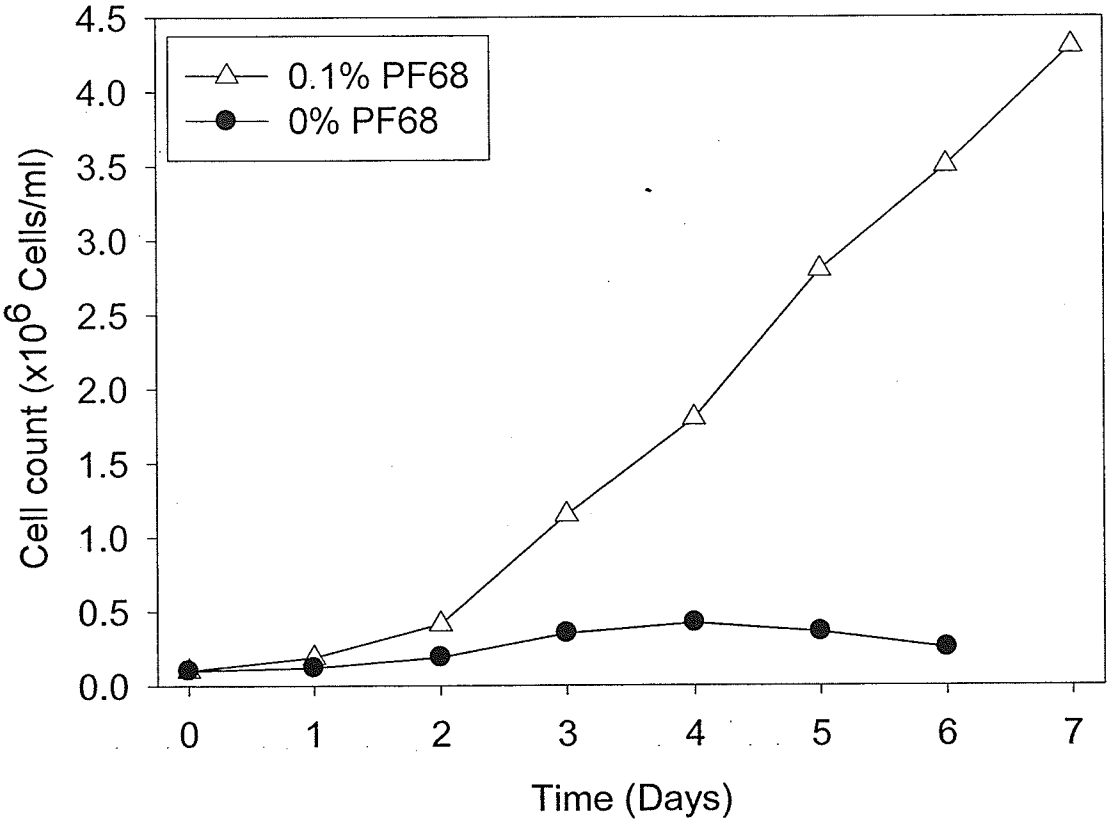


Figure 9.3: The effect of PF68 in agitated cultures. IFN-beta producing CHO cells were grown in a 2 L bioreactor for up to 7 days in the presence of PF68 (△) or absence of PF68 (●), following 3 passages in PF68-free medium. The marine impeller rotation speed was 100 rpm with sparging to keep the dissolved oxygen level at 50 %. Viable cell concentration was estimated using the trypan blue exclusion method(0.5 % w/v).

9.2.2.1. Spinner flasks/ high agitation

Figure 9.4 shows the percentage of viable cells remaining after exposure to a high agitation rate of 500 rpm over a period up to 5 h. In this experiment the two CHO cell lines (producing IFN-beta and tPA, respectively) were grown in PF68-free medium for 3 passages prior to inoculation into spinner flasks. The left (Figure 9.4A) and right (Figure 9.4B) panel represents CHO cells producing IFN-beta and those producing tPA, respectively. The cells were seeded into the spinner flasks at $1x10^6$ cells/mL before agitation at 500 rpm for 5 h. The cell viability of the cultures was determined from samples taken at 30 min intervals. From the data, there was no evident overall loss in cell viability of either of the cell populations in the presence of PF68, with the cell viability remaining close to 100 %. However, there was significant and rapid cell damage in the cultures that were exposed to the high agitation rate in the absence of PF68. This resulted in large clumps of cellular debris that was evident in each culture.

There was a significant difference in the rate of damage observed for the two cultures ($p<0.0001$ in both cases). The IFN-beta cell line was reduced to zero viability within 1 h. However, the viability of tPA producing cell line decreased rapidly during the first 30 min followed by a more gradual phase of decrease up to the 5 h point. This difference suggests an intrinsic difference in robustness of the cell lines in response to shear stress.

Most experiments explained here were done in PF68 at 0.1 % which is a concentration commonly used in culture media. Figure 9.5 shows the result of cells being agitated in a shaker flask at different concentrations of PF68. The cells ($10x10^6$ cells in 5 mLs) were taken from T-flask cultures growing at 0 % PF68 for 3 passages then

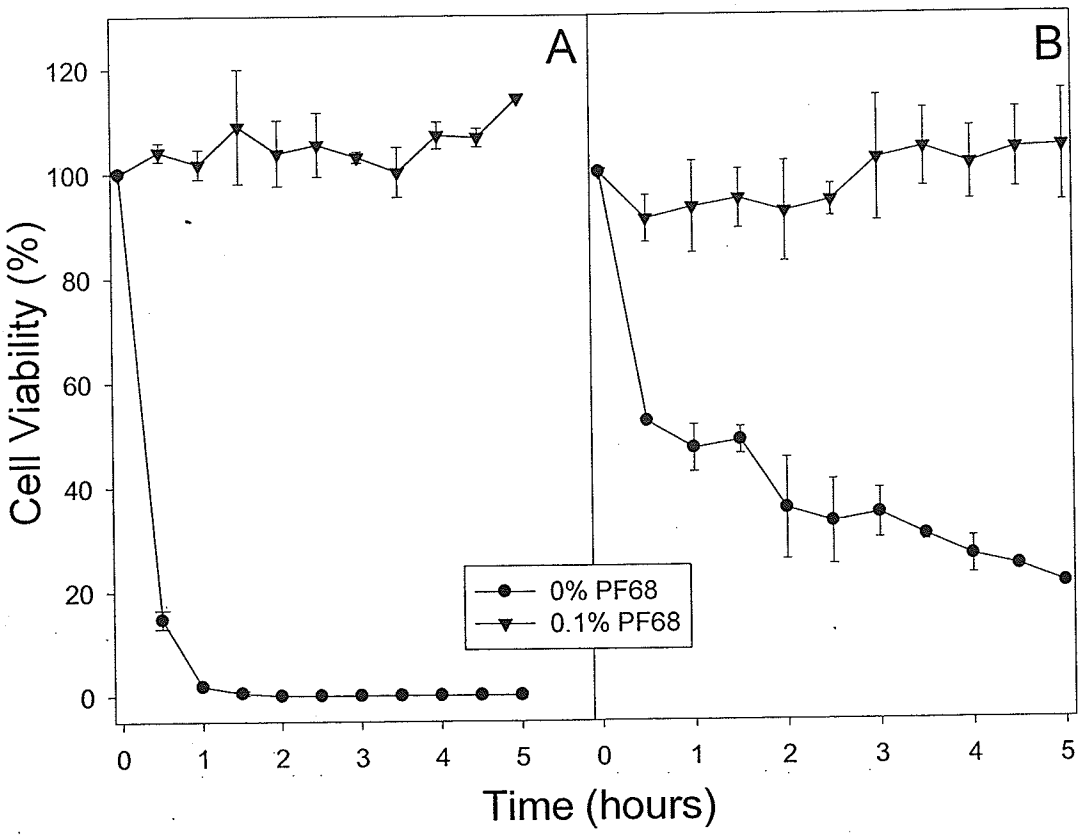


Figure 9.4: Cell sensitivity to high speed agitation. CHO cells producing (A) IFN-beta and (B) tPA were grown in the absence or presence of PF68, following 3 passages in PF68-free medium, and were subjected to a high rotation speed of 500 rpm in a 100 mL spinner flask for up to 5 hours. Cell viability was estimated using the trypan blue exclusion method (0.5 % w/v) and expressed as a mean (±SEM) where (n=2) for cultures in the absence of PF68 (●) or in 0.1 % PF68 (▼).

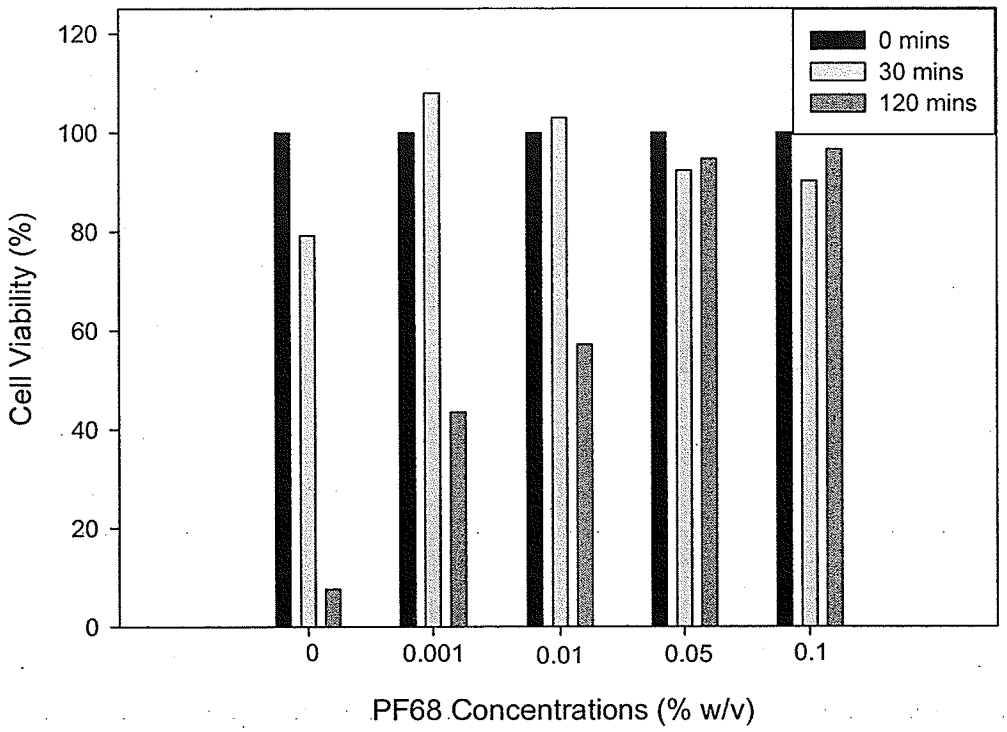


Figure 9.5: Cell sensitivity to high speed agitation in shaker flasks. CHO cells were grown in the absence of PF68 for 3 passages and then exposed to high rotation speeds of 300 rpm in the different concentrations of PF68. Samples were counted at 0, 30 and 120 mins. Cell viability was was estimated using the trypan blue exclusion method (0.5 % w/v).

the cells were agitated at 300 rpm for up to 2 hours. In the absence of PF68 the cell viability in 0 % PF68 dropped to 80 % after 30 minutes of agitation. Furthermore after 2 hours the cell viability was 7.6 %. The protection of cells from the shear forces from the agitation seemed dependent on concentration of PF68. The cells in 0.1 % and 0.05 % PF68 retained a viability close to 100 % during the assay. The viability of cells in 0.001 % PF68 and 0.01 % was 44 % and 57 %, respectively after 120 minutes of agitation.

9.2.2.2. Shear stress in a bubble-free environment

Although designed for the measurement of liquid viscosity, the cone-and-plate viscometer offers a means of subjecting cells to a shear force caused by a high relative rotational velocity between two metal plates. Gas bubbles can be removed from the space in which the liquid is introduced, thus eliminating any gas-liquid interface. Several commercially-available viscometers were tested for their suitability. The Rheolab MC1 was chosen because it produced a high shear rate of up to 11,000 s^{-1} which was sufficient to produce a complete decay curve of cell viability within 10 min and with a small sample volume of up to 600 μL.

Samples (350 μL) from cultures of two CHO cell lines (IFN-beta and tPA producing) grown in the absence of PF68 for 3 passages, were subjected to a shear force of 11 Pa for a period up to 12 min in the presence or absence of PF68. The cell viability of each sample was determined after the period of exposure to the shear force (Figure 9.6).

The results clearly show the protective effect of PF68 (0.1 %) in this system with viabilities maintained >90 %. In the absence of PF68 the viability of the two cell lines

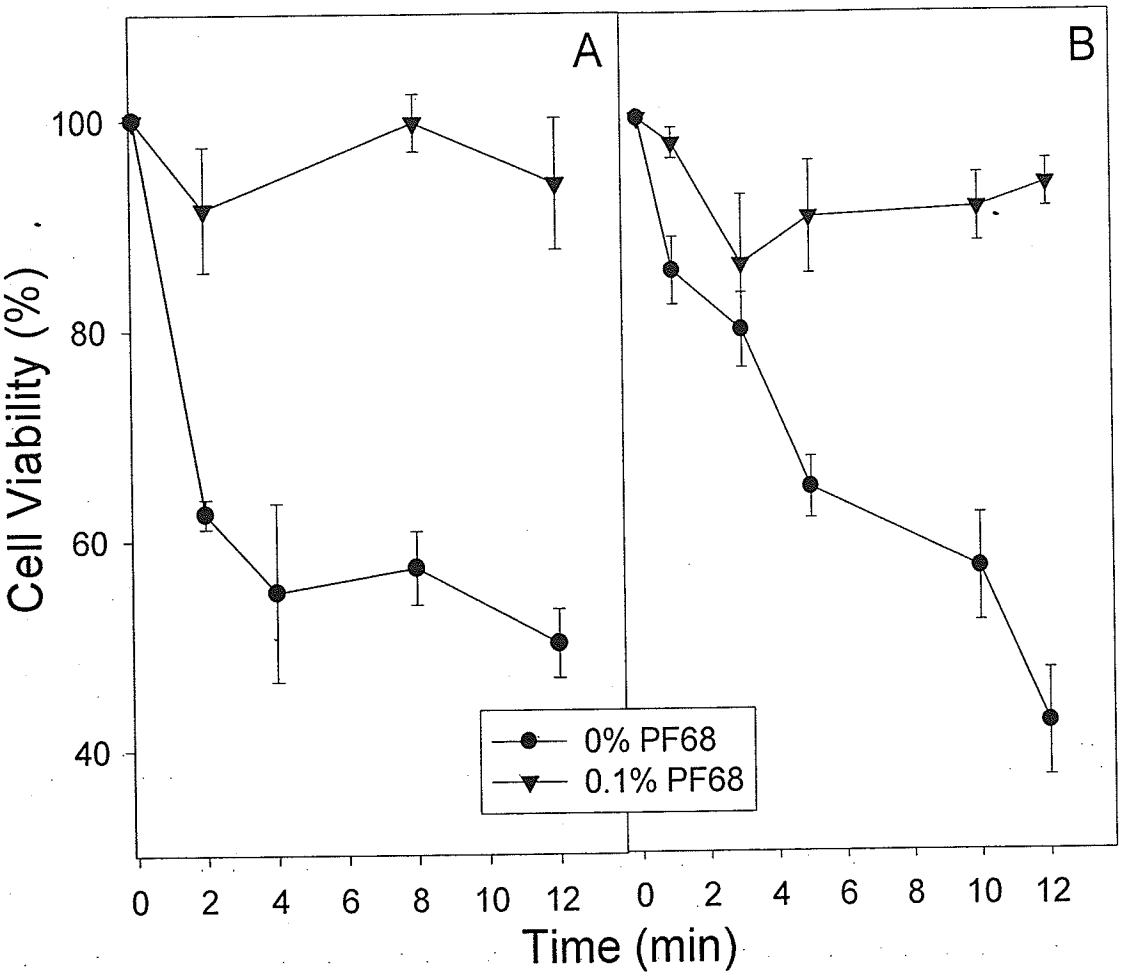


Figure 9.6: The effect of high shear rates in a bubble-free system. CHO cells producing (A) IFN-beta and (B) tPA were grown in the absence (●) or presence (▼) of PF68 were subjected to a shear rate of 11,000 s^{-1} in a bubble free environment provided in a cone and plate viscometer for up to 12 minutes. Cell viability was estimated using the trypan blue exclusion method and expressed as a mean (±SEM) where $n \geq 3$.

decreased to values between 40 to 50 % after 12 minutes. The rate of decrease of cell viability appears to be lower for the tPA producing cell line (Figure 9.6B) and suggests the enhanced robustness of this cell line as previously inferred from the spinner flask results. However, there does appear to be a residue of viability in the IFN-beta producing cell line (Figure 9.6A) after 12 min that seems to be resistant to further rapid decay. This is unexplained but may be due to aggregation of cells or the presence of cell debris.

9.2.3. Long term effects of PF68 on cell protection

In order to determine whether the presence of PF68 during cell growth would affect the long-term robustness of cells, cells were grown in the presence of PF68 then exposed to a shear force in the absence of PF68. Cells were grown in stationary cultures in the presence of varying concentrations of PF68 (0-0.1 %). The cells were then harvested and resuspended in medium with no PF68 at a cell density of $2x10^6$cells/mL. The cell suspensions were then exposed to a shear rate of 11,000 s^{-1} for 10 minutes. In each case the cell viabilities decreased to 50 %, with a profile that was not significantly different from that of cells previously grown and tested in the absence of PF68. This result indicates that PF68 does not have a continued long-term effect of shear protection once removed from the culture (Figure 9.7).

The protection of PF68 after shear force exposure is shown in Figure 9.8. Cells were grown in PF68-free media for 3 passages were exposed to shear in the viscometer. The cells were then exposed to PBS with and without PF68 for 2 minutes, then enumerated

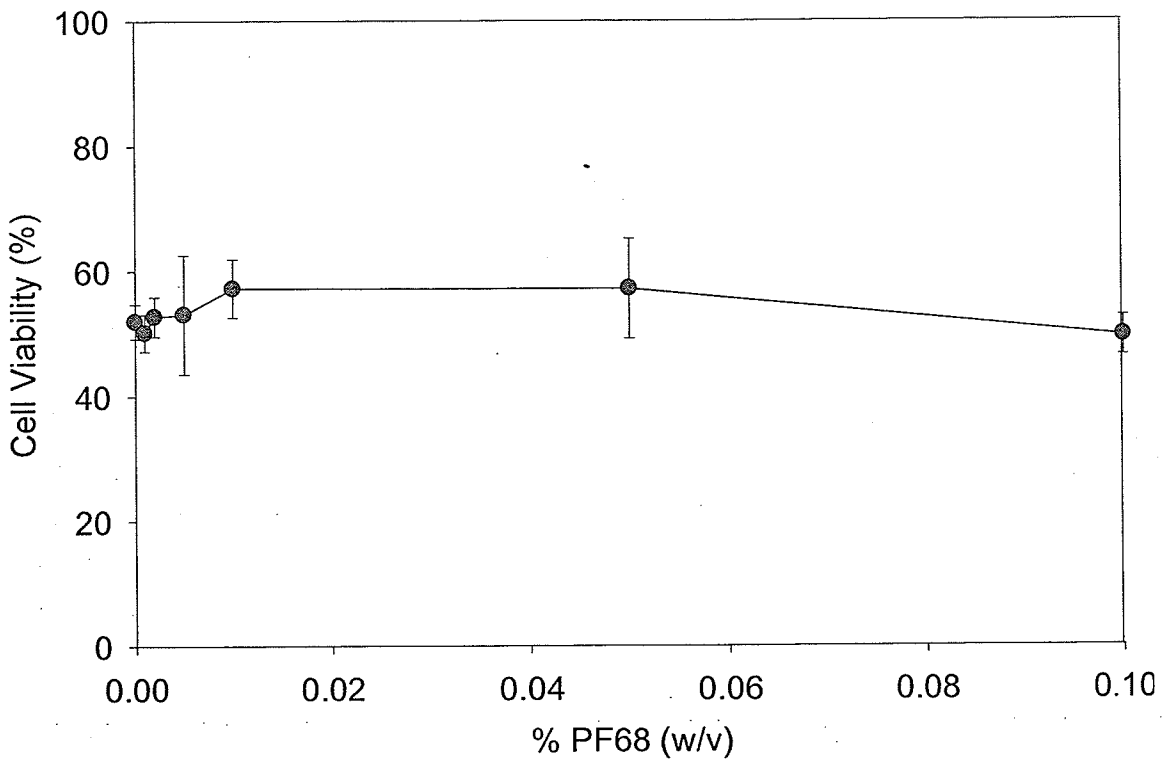


Figure 9.7: Long term effects of PF68 on shear stress by a viscometer. Cells grown in media containing varying concentrations of PF68 were transferred to PF68-free media prior to being treated in the viscometer at 11,000 s^{-1} for 10 minutes. The values are expressed as means (± SEM) where $n \geq 3$.

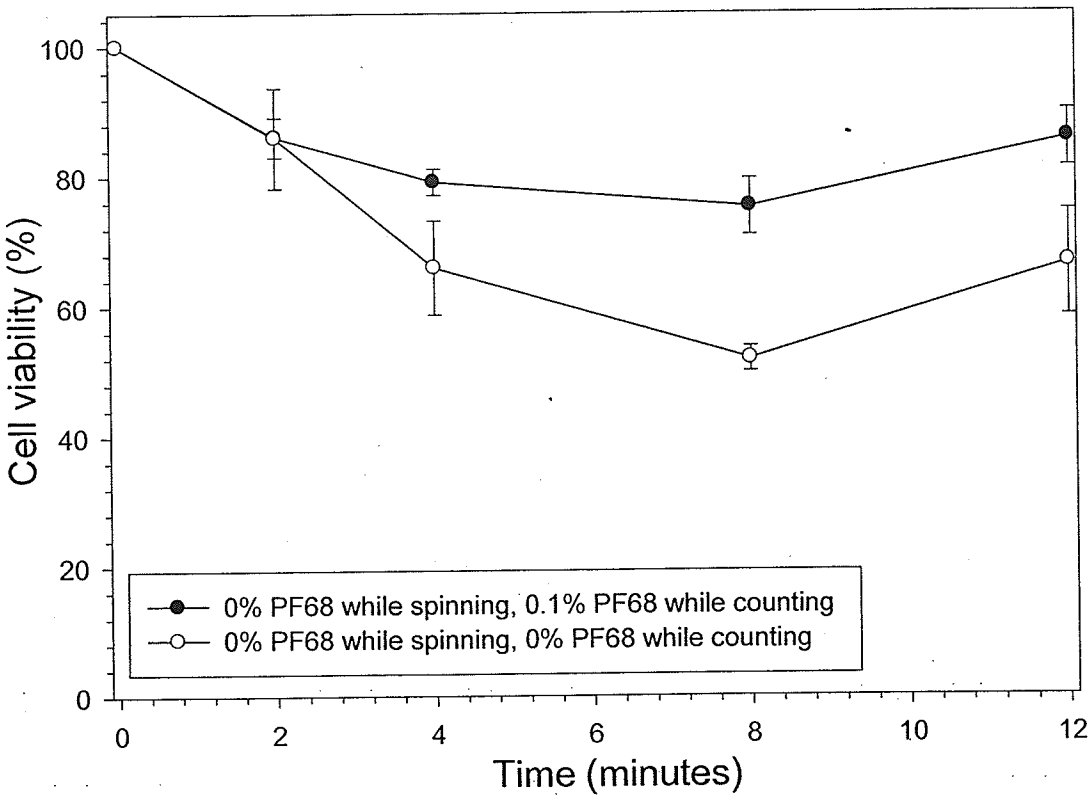


Figure 9.8: The protection of cells by PF68 after shear force exposure. Cells were exposed to shear forces in the viscometer in the absence of PF68. PF68 additions were made to the cell samples prior to the enumeration of cells using the trypan blue exclusion method. Samples represent an average of n=5 ± SEM.

via the trypan blue exclusion method. Cells that were exposed to shear and enumerated in the absence of PF68 showed a lower cell viability (52 %) after 8 minutes compared to the sample in the presence of PF68 (80 %).

The higher cell viability may be due to the interference of PF68 with the counting method. Thus we followed a similar protocol to enumerate cells that were not exposed to shear forces to see if the difference in the cell viability was an artifact. The cells were divided into two fractions of $3.0x10^6$ cells/mL in PF68-free media, and then each was diluted with media either containing 0.2 % PF68 or 0 % PF68. Cell samples were counted at 1, 5, 30 and 60 minutes post dilution by trypan blue exclusion. The results are as shown in Figure 9.9. Under both conditions of counting, the cell viability remained close to 100 %, thus indicating that there was no interference of PF68 in the counting method.

It was also determined if PF68 would protect cells from the shear forces induced by pipetting prior to enumeration. Thus an assay was developed to see if multiple pipetting steps caused a decrease in cell viability. The cells were grown in 0 % and 0.1 % PF68 and then resuspended in media with and without PF68 before estimated using the trypan blue exclusion method. The mixture was then resuspended using a micropipette (150 µl) up and down for varying times. The final counts after resuspending were compared to the initial to get cell viability, which is as shown in Figure 9.10.

The results of this experiment show that the presence of PF68 could protect cells from shear forces during multiple pipetting steps. The cells that were grown in the presence of PF68, then treated to shear force by pipetting in the absence of PF68 showed a decrease

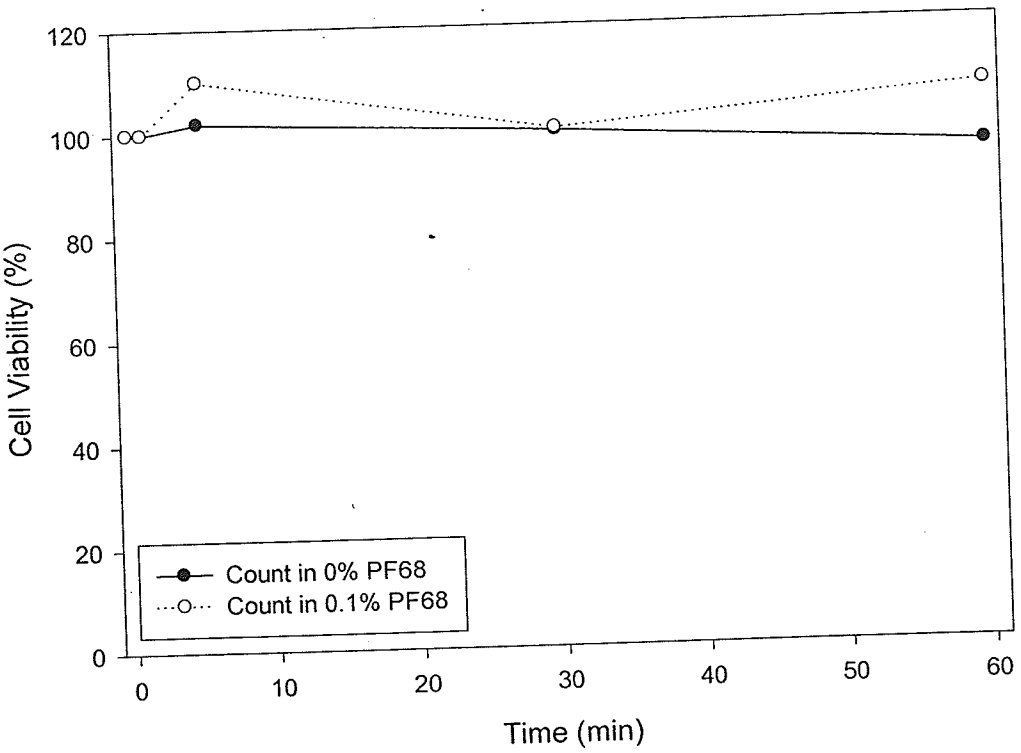


Figure 9.9: The lack of interference of PF68 during cell enumeration by the trypan blue exclusion method. The cells were divided into two fractions of 3.0×10^6 cells/mL in PF68-free media, and then each was diluted to 0.1 % PF68 (○) or 0 % PF68 (●). Cell samples were counted at 1, 5, 30 and 60 minutes and compared to initial counts. The values reported are representative of typical results observed.

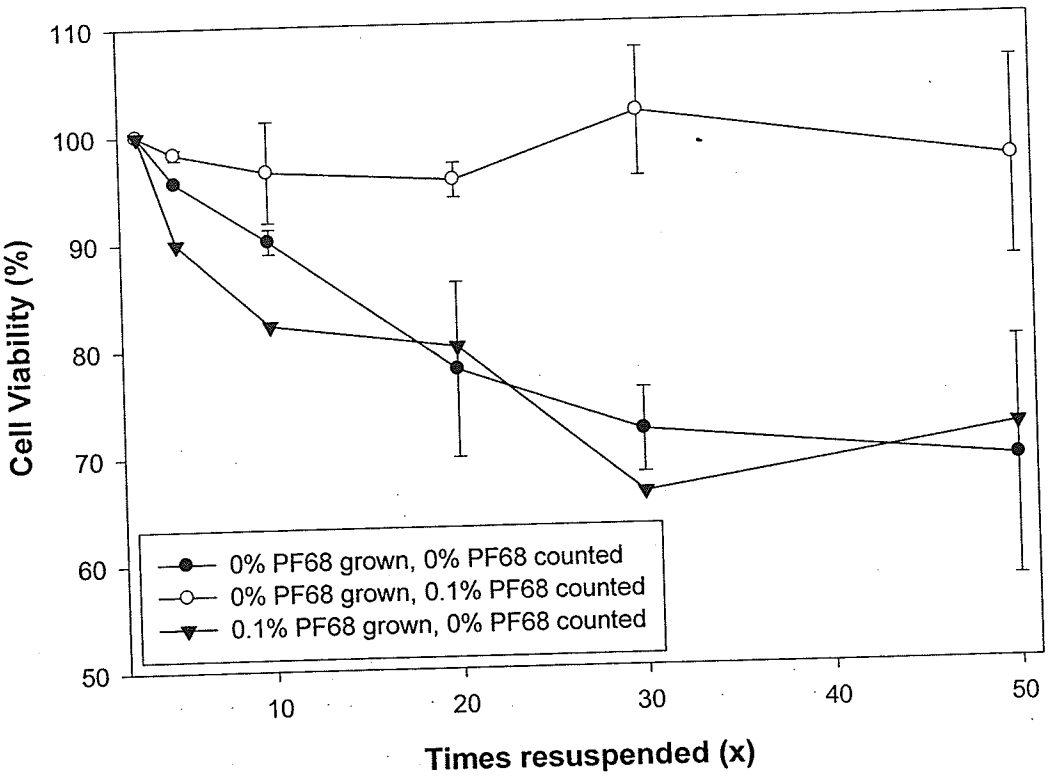


Figure 9.10: The effect of repeated pipetting on cells grown in the absence of PF68 (●/○) and the presence of PF68 (▼). The cells grown in the absence of PF68 were resuspended in media with PF68 (○) and without PF68 (●) before being enumerated using the trypan blue exclusion method. The samples are an average of n=3 ± SEM.

in cell viability to 70 % after 50 aspirations in the micropipette. This was also true for the cells grown and pipetted multiple times in the absence of PF68, where the cell viability decreased to 70 %. The cells that were grown in the absence of PF68 then exposed to pipetting in the presence of PF68 had a cell viability of 100 % throughout the assay. Thus this experiment showed that cells are protected by shear forces in the presence of PF68, and that there is no long term protection offered by PF68.

9.2.4. Trypsin

Trypsin is used routinely in cell culture to remove adherent cells from culture surfaces or to disaggregate clumped cells. However, it is generally believed that membrane damage may be caused by prolonged exposure of cells to the proteolytic enzyme. In this experiment, we aimed to test the ability of PF68 to repair the damaging effect of trypsin. The cells were exposed to trypsinization times (>10 min) which are greater than those generally used in practice, in order to test the ability of PF68 to ameliorate the potentially damaging effects on the membrane.

Preliminary experiments were performed to see the effects of trypsin on the cells. Cells were incubated in suspension in PBS containing trypsin (0.05 % w/v) for up to 300 minutes at 37°C. At different time intervals cells were removed from the sample and enumerated as a percentage of the original counts. Figure 9.11 shows the results from this experiment. Briefly, as the cells are incubated with this proteolytic enzyme, the cell viability decreases. At 60 minutes the cell viability reached 57 % and this was consistent until the end of the assay at 240 minutes.

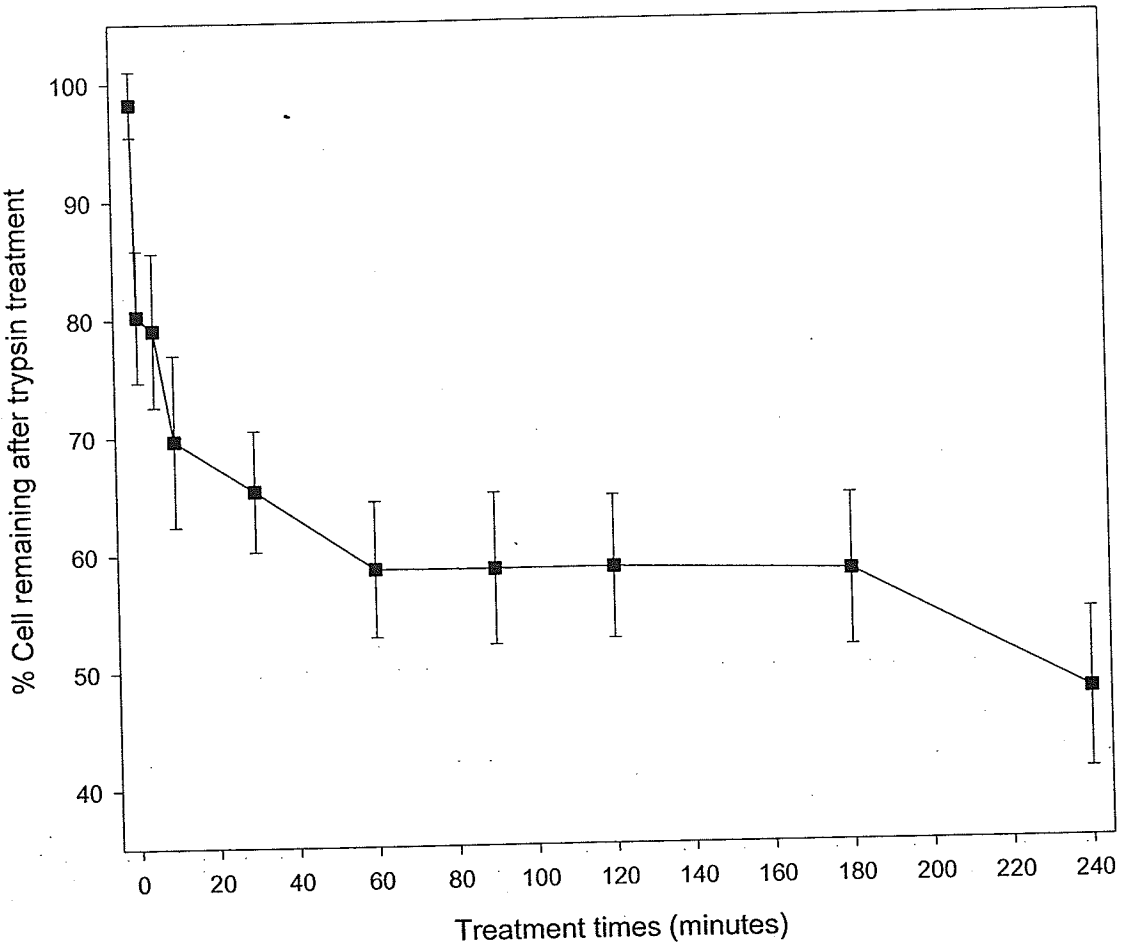


Figure 9.11 The effect of trypsin on cells in PBS from 0 to 240 minutes. The cell density was estimated using the trypan blue exclusion method. Samples represent an average of n=8 ± SEM.

The addition of PF68 during trypsinization appears to decrease the cell damage due to the proteolytic activity, and is shown in Figure 9.12. The trypsinization of cells in the presence of PF68 shows no effect on the cell viability, where during the trypsin incubation period the cell viability remains close to 100 %. However, in the absence of PF68 the cell viability drops 10 % after 30 minutes and 40 % after 60 minutes of incubation with trypsin. Thus this experiment shows the protective effect of PF68 on the proteolytic enzyme activity of trypsin.

The possibility of an overestimation of cell viability caused by PF68 preventing trypan blue uptake by non-viable cells during the assay was discounted through a control. Here trypsin-damaged samples of cells that had not been in contact with PF68 were serially diluted. Viable cell count at each dilution was measured by the trypan blue method with and without the presence of 0.1 % PF68. In accordance with the standard assay protocol, the viable cells were counted within 5 minutes of trypan blue addition. The results showed that under these conditions there was no significant difference in the two sets of viable and non-viable counts, showing that there was no effect of the presence of PF68 in the counting assay (data not shown).

This protective effect was also evident when treating trypsinized cells to high shear forces in the cone-and-plate viscometer. Figure 9.13 shows the residual viable cells remaining after various treatments. Cells treated with trypsin and then subjected to shear stress in the viscometer in the absence of PF68 showed the greatest decrease in viability with a residue of only 48 % viable cells. Significantly higher yields of residual viable cells were found if the cells were not exposed to trypsin (62 %; $p=0.02$) or if PF68 was

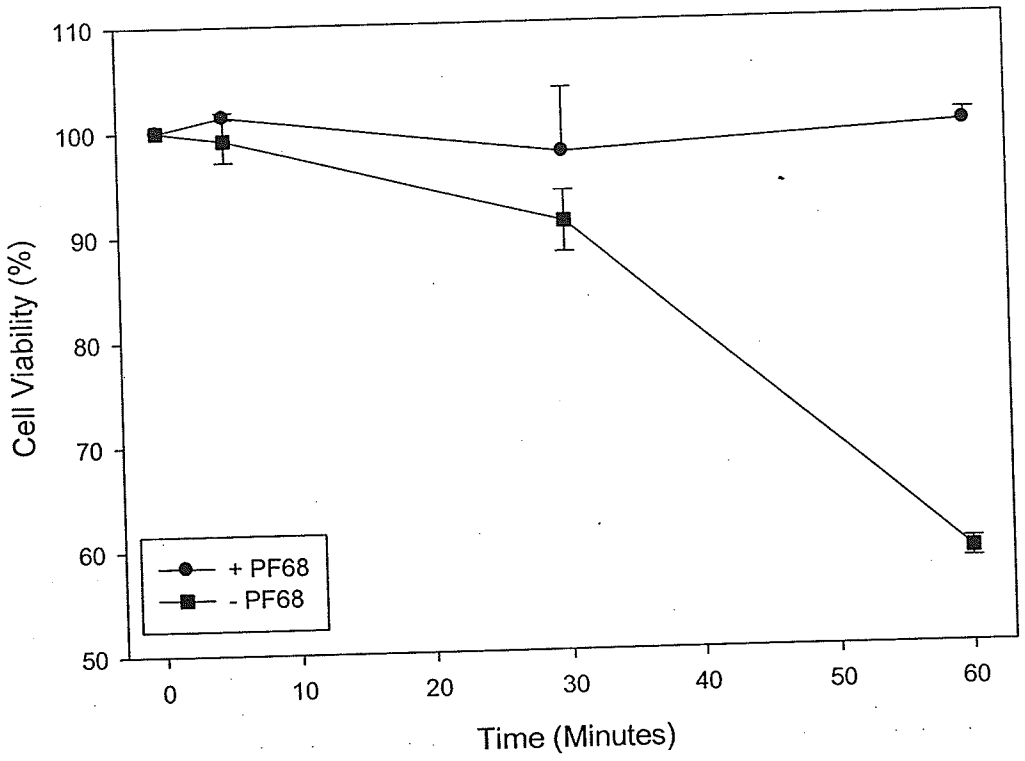


Figure 9.12: The trypsinization of cells in the presence of PF68. The cells were enumerated by using the trypan blue exclusion method. The samples represent n=2 ± SEM.

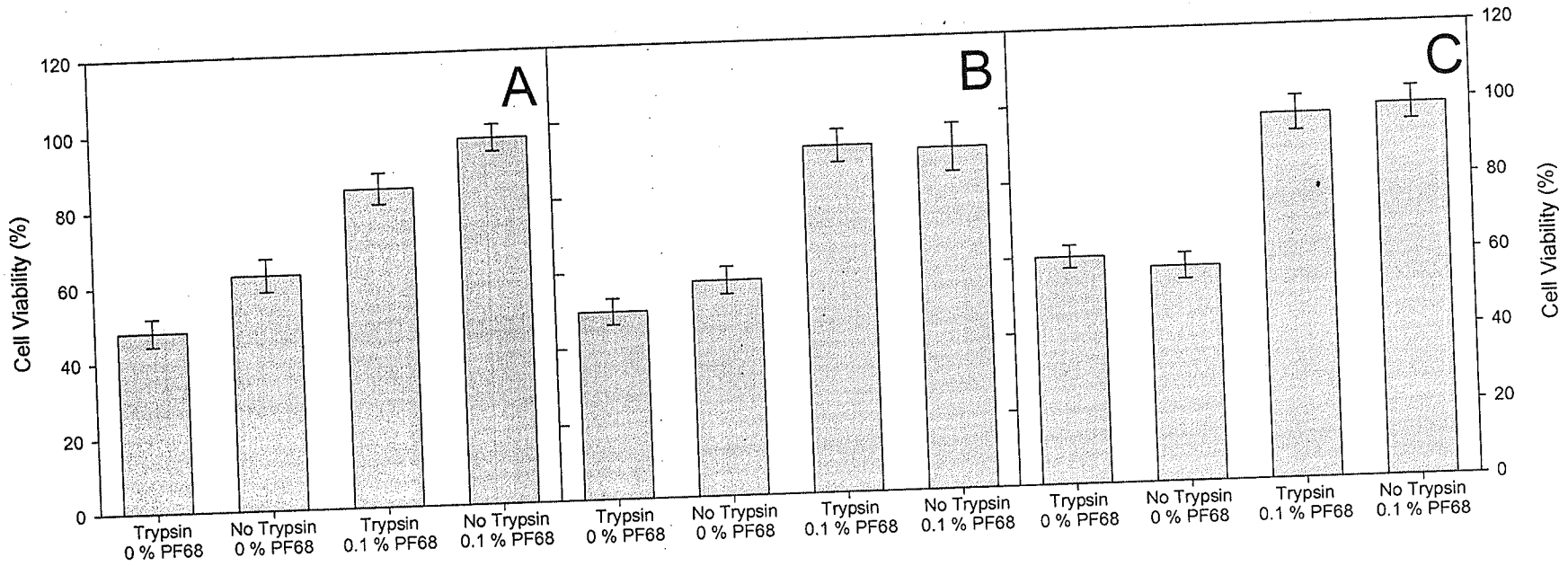


Figure 9.13: Shear sensitivity of CHO cells exposed to trypsin after passage 1 (A), passage 2 (B) and passage 3 (C). Cells (IFN-beta producing) were grown in agitated culture in the absence of PF68 prior to harvesting by centrifugation. Samples of the cells (5 mL at $2x10^6$ cells/mL) were treated with trypsin (0.5 % w/v) for 10 min (condition 1 and 3). Each cell suspension (350 µl) was then exposed to a shear rate of 11,000 s^{-1} in the cone-and-plate viscometer for 12 minutes in the presence (condition 3 and 4) or absence (condition 1 and 2) of 0.1 % PF68. The values shown are means (±SEM). (n≥3).

added to the cell suspension prior to the application of shear stress ($p<0.00002$). Furthermore, the addition of PF68 to trypsin-treated cells enhanced the residual cell viability.

This difference between shear sensitivity was evident in the first passage of cells being trypsinized in the absence PF68. Cells from the second and third passages after trypsinization appeared to show no difference in shear sensitivity due to trypsinization. The sensitivity was evidently due to the exposure of shear stress in the presence or absence of PF68. Cells that were either trypsinized or not trypsinized but exposed to shear forces in the absence of PF68 showed no significant difference in the percent viable cells remaining after shear stress exposure ($p=0.5$) (data not shown). There is no valid explanation for the difference in the cells reaction to shear forces when comparing the cells reaction to trypsinization in the first passage to that in the following passages. However, it is possible that the cells in the subsequent passages may have been selected out by trypsinization, and thus are a trypsin resistant cell population.

A further experiment was designed to determine if PF68 could provide a protective barrier to the membrane of damaged cells. In this experiment cells ($2x10^6$ cells/mL) were incubated with trypsin (0.5 % w/v) for varying lengths of time up to 4 h. The activity of the trypsin was stopped by the addition of trypsin inhibitor (0.09 % w/v). The cells (1 mL) were then suspended in PBS with or without PF68 for 30 min prior to cell counting using the standard trypan blue assay. The results are shown in Figure 9.14 and indicate that the proportion of trypan blue-stained cells increased significantly with the time of exposure to trypsin. This showed that trypsin had an effect on the increase in membrane

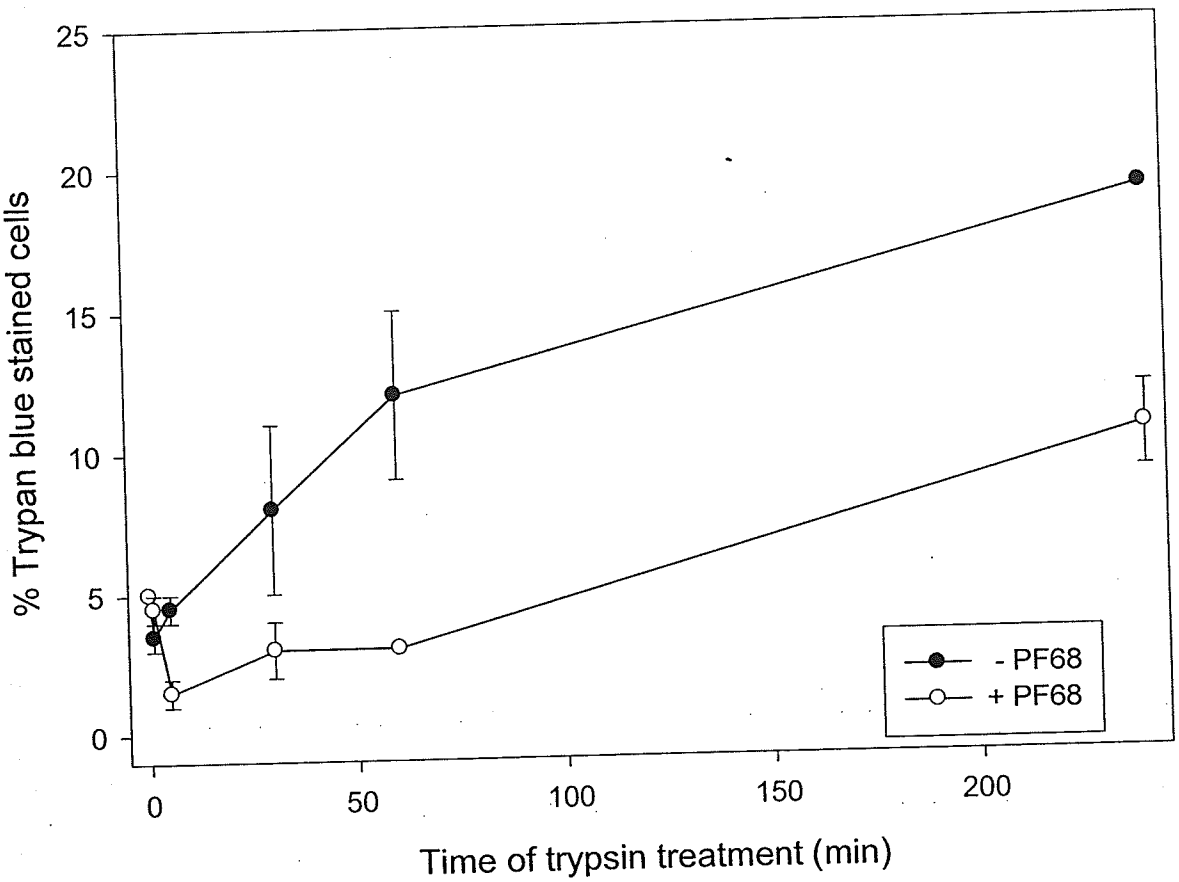


Figure 9.14: The protective effect of PF68 upon trypsin-damaged cells. IFN-beta producing cells (1 mL at $2x10^6$ cells/mL) were incubated with trypsin (0.5 % w/v) for times up to 240 min at 37°C. The enzymatic activity was stopped by the addition of trypsin inhibitor (0.09 % w/v). A portion of each cell suspension (0.5 mL) was then re-suspended in PBS in the presence of absence of PF68 (0.1 %) for 30 minutes prior to staining in trypan blue (0.5 % w/v) for counting in a haemocytometer. The proportion of blue-stained cells was recorded in each sample for suspension containing PF68 (○) or in the absence of PF68 (●). The values shown are means (±SEM). (n=2).

porosity of all the cells tested. However, the addition of PF68 to the trypsin-treated cells prior to the exposure to trypan blue reduced the uptake of the dye significantly. This was particularly evident for the cells treated with trypsin for longer than 60 min when the proportion of stained cells was up to 10 % less in the presence of PF68 ($p<0.01$). This indicates that PF68 significantly decreased the membrane porosity of cells damaged by trypsin.

In a separate series of experiments the effect of PF68 was determined on the growth of trypsin-damaged cells. Cells were inoculated at $1x10^5$ cells/mL into 100 mL spinner flasks and grown for 4 days. The harvested cells were then suspended in PBS-EDTA (100 mL), and dispensed equally into 2 centrifuge tubes prior to centrifugation. One cell pellet was then incubated in trypsin (0.5 %) in PBS-EDTA for 120 minutes, while the second pellet was incubated in PBS-EDTA. The suspensions were agitated intermittently to maintain the cells in suspension. After incubation trypsin inhibitor (0.09 % w/v) was added and the cells were inoculated at $1x10^5$cells/mL into 100 mL spinner flasks in cultures with or without PF68. The corresponding growth curves of these cultures are shown in Figure 9.15.

Figure 9.15A shows the growth of the CHO cells in the absence of PF68. The control culture that was not exposed to trypsin showed a steady growth rate after day 1 to a maximum cell density of $1.7x10^6$ cells/mL. However, the damaging effect of trypsin exposure in the second culture is seen by the significantly lower growth rate and maximum cell density of only $0.6x10^6$ cells/mL ($p<0.005$). Figure 9.15B shows the effect of PF68 on these cells. Whereas, there is no considerable difference in the growth

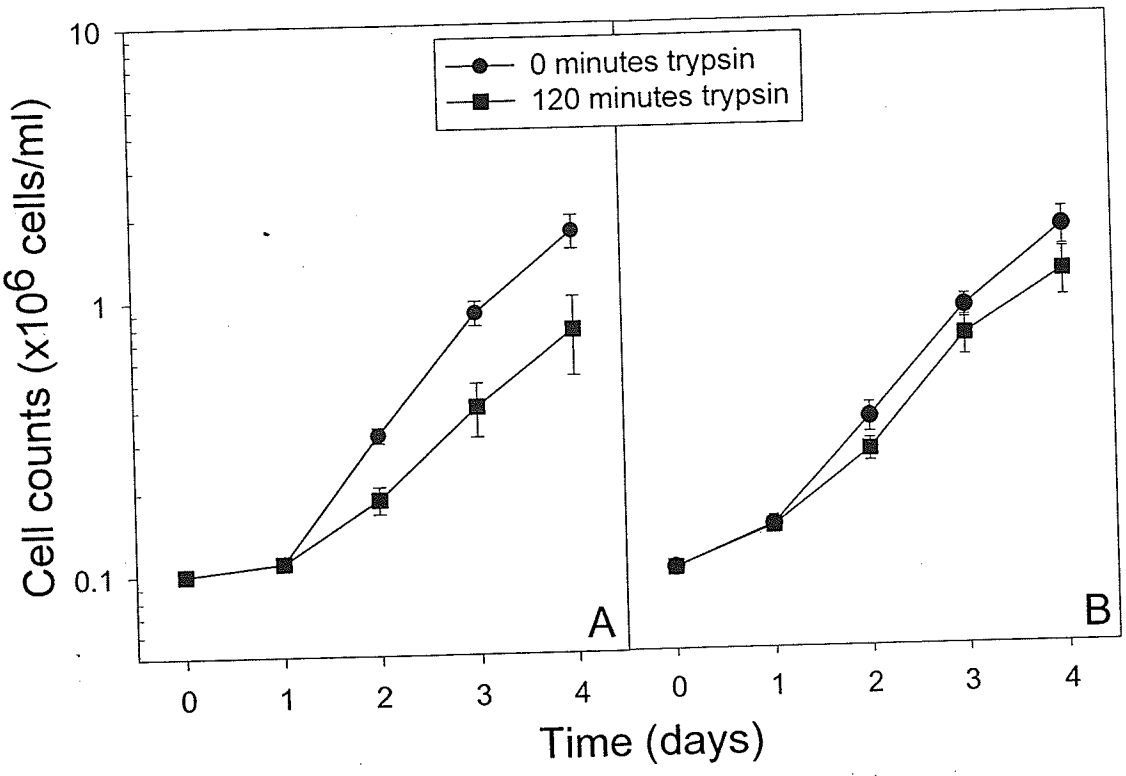


Figure 9.15: The effect of trypsin damage on cell growth. Twenty five mL of IFN-beta producing CHO cell suspension at $2x10^6$ cells/mL were trypsinized (0.5 % w/v) for 120 minutes which was then inhibited using soybean inhibitor (0.9 % w/v). A control cell sample was incubated in PBS-EDTA for 120 minutes. The trypsinized and non-trypsinized cells were then inoculated at $1x10^5$ cells/mL into a PF68-free cultures (panel A) or 0.1 % w/v PF68-containing cultures (panel B) in 100 mL spinner flasks and grown over a 4 day period at 37°C. The values are mean cell counts (±SEM) where ($n \geq 3$) for trypsinized cells (■) or controls (●).

profile of the control cells that were not exposed to trypsin, PF68 appears to have a significant effect on the trypsin-exposed cells ($p<0.005$). In this case the higher growth rate results in a maximum cell density of $1.2x10^6$ cells/mL at day 4, which is twice the yield of the equivalent culture grown in the absence of PF68.

9.3. Discussion

PF68 has been used as a supplement for mammalian cell cultures for a number of years as a cell protectant particularly in scale-up of stirred-tank bioprocesses (Kilburn and Webb, 2000). It has a well-characterized function as a cell protectant against the shear forces that arise from impeller agitation and bubble dispersion during gas sparging. This requirement for such protection is even greater in the low protein or protein-free environment of current serum-free media formulations that expose cells to harsher physico-chemical conditions than the more traditional high protein serum-based formulations previously used.

Cell destruction in sparged, agitated cultures is likely to be due to cell-laden bubbles drawn into the liquid-gas interface where bubble disengagement occurs (Michaels 1995). This zone at the gas-liquid interface is where the cells are subjected to forces that cause lysis (Kunas and Papoutsakis, 1990).

Several mechanisms have been suggested for the protective effect of PF68 in agitated cell cultures. One is that PF68 reduces the interaction between cells and bubbles that arise from sparging by a decrease in cell- to-bubble attachment (Jordan *et al.*, 1994; Meier *et al.*, 1999; Michaels *et al.*, 1995; Zhang *et al.*, 1992). This mechanism explains

the growth profile we observed in a sparged bioreactor, in which there was negligible growth of CHO cells in the absence of PF68. This mechanism could also explain the concentration-dependent protective effect of PF68 at high agitation rates in spinner cultures (500 rpm) and shake flasks (300 rpm). This assay system was developed previously in our laboratory to measure the robustness of cells following treatment with selective media components such as linoleic acid (Butler *et al.*, 1999). Agitation rates above 250 rpm in the spinner flasks produce an observable liquid vortex, which introduces bubbles into the medium. Therefore the protective mechanism offered by PF68 could be similar to that seen in the sparged bioreactor. This assay showed differences in the intrinsic robustness of both of the CHO cell lines tested, the IFN-beta cell line showed a rapid death rate with a complete loss of viability after 1 hour, whereas the tPA producing cell line showed a more gradual decline with a residual of 20 % viability even after 5 hours.

PF68 may also increase the cell membrane resistance to liquid shear in the absence of bubbles or a gas-liquid interface. To test this we subjected both the cell lines to an assay based on the use of a cone-and-plate viscometer producing a maximum shear rate of 11,000 s^{-1} , in which there was a rapid loss of cell viability over a 12 min test period. Results from this assay showed that there was a significant protective effect of PF68 on the cells in the bubble-free environment of the viscometer. Marquis *et al.* (1989) reported some protection of cells in a coquette flow system with a lower shear rate of 6,000 s^{-1} . Further evidence for the protective effect of PF68 in liquid shear arises from the work of Zhang *et al.* (1992) who showed by micromanipulation techniques that the critical tension required to burst hybridoma cells increased significantly in the presence

of PF68. Goldblum *et al.* (1990) reported the same effect on insect cells using a modified rheogoniometer, which is a device similar to viscometer but with a rotating plate and a stationary cone.

This high shear assay also indicated differences in the intrinsic robustness of different cells with half-lives of around 5 min for the IFN-beta producing cell line, whereas the tPA producing cell line showed greater resistance with a more gradual decay curve and a half-life of 10 minutes.

The difference in the sensitivity of cells to shear can be due to the intrinsic difference in the cell membrane, cytoskeleton or due to a difference in the size of cells. Our lab has previously reported differences in the hydrophobicity of the cell surface of hybridoma and CHO cells (Ghebeh *et al.*, 2002). Furthermore CHO cells have been shown to have a ruffled surface, depending on the method of culture, compared with hybridoma cells with a smooth cell boundary. This is evidence for an intrinsic difference in the nature of the membranes of the two different cell lines. In this study, both the IFN-beta and tPA producing CHO cells took on a ruffled appearance. Zhang *et al.* (1995) reported that 0.1 % (w/v) PF68 enhanced erythrocyte cell lysis in flasks agitated at 100 rpm, while 0.1 % (w/v) dextran or albumin prolonged erythrocyte survival. Larger cells are more prone to shear forces than smaller cells (Al-Rubeai *et al.*, 1995). Under the microscopic examination and using a haemocytometer the CHO cells were observed to be x1.5 larger in diameter than hybridoma cells. The observed diameter range of CHO cells was 15 to 25 microns compared to hybridoma cells with diameter range of 10 to 15 microns.

Although in our experiments cells were protected from high shear rates only when PF68 was present in the assay. In general, differences in the intrinsic cell resistance to shear may also be due to a change in the plasma membrane fluidity (Calder *et al.*, 1994). Although we have no evidence that PF68 could alter the membrane fluidity this possibility is supported by Ramirez and Mutharasan (1990), who suggest that PF68 may interact directly with the membrane structure enhancing its stability. This suggests the possibility of prolonged protection by PF68 that has been shown previously in insect cells (Murhammer and Goochee, 1990). There was no evidence for long-term stabilization of CHO cells by PF68 in our culture systems. These apparent differences may be because of variability between cell lines or due to the metabolic state of the cells.

Wu *et al.* (1996) reported that PF68 could decrease the hydrophobicity of insect cell membranes. In a recent paper we also showed that PF68 decreases the cell hydrophobicity index of CHO and hybridoma cells (Ghebeh *et al.*, 2002). It is suggested that a reduction in the hydrophobicity index of the surface of cells accounts for the decreased cell-to-bubble attachment in the presence of PF68. Murhammer and Goochee (1990) investigated the properties of different molecular structures of the family of Pluronic polymers and showed that the hydrophilic/ lypophilic balance is an important criterion for predicting their ability to protect cells in culture, which suggests their interaction with the cell membrane. Furthermore Wu *et al.* (1997) measured the adsorption of PF68 molecules to the cell membranes by measuring the decrease in the PF68 concentration in PBS after mixing with insect cells. Al-Rubeai *et al.* (1993) reported that PF68 could block a leaky cell membrane and protect surface-associated

immunoglobulin in hybridomas from shear damage. Lakhotia *et al.* (1993) found that PF68 can protect CD33 surface antigen of HL60 human leukemia cells from shear forces.

One of the most likely explanations for the effects of PF68 is that it is adsorbed on to the membrane surface creating an artificial wall that may protect cells and even repair damaged cells. This is supported in our experiments by the evidence of enhanced shear resistance, decreased membrane porosity and higher growth rates of cells in PF68-supplemented medium that have been damaged by the effects of prolonged incubation in trypsin. The effect of trypsin in the presence of PF68 is most likely to be due to a protective effect, and not inactivation of trypsin by PF68. The trypsinization of cells in PBS requires inactivation by a trypsin inhibitor when SFM is used in cell culture (Cruz *et al.*, 1997). Resuspending cells in serum containing media does not require this inactivation step, as there are natural inhibitors in serum that would inactivate this proteolytic enzyme.

The sensitivity of CHO cells to shear forces has an important implication to the development of bioprocesses that includes the design of the impeller, the agitation speed and the intensity of sparging. PF68 supplementation in culture has many advantages that can reduce the deleterious effects of both gas sparging and liquid shear caused by culture agitation as well as maintaining a single cell suspension. However, there are offsetting disadvantages of using PF68. These include the difficulties of removing PF68 during product recovery as well as the potential inhibition of growth for some cell lines (Al-Rubeai *et al.*, 1992). For these reasons it may be important to minimize the concentration of PF68 in the culture. As the sensitivities to the shear forces in culture can differ

between cell lines, this minimal concentration of PF68 should be determined for each cell line.

The protection by PF68 is not limited to mammalian cells, as plant cells were protected by high shear forces in the presence of PF68. In particular, a pepper cell culture was protected by the phenols produced by the cells when PF68 was added into culture medium (Kaparakis and Alderson, 2003). Furthermore the effects of PF68 on the cell protection were further enhanced with the combination of haemoglobin and arabinogalactan proteins.

In conclusion we have shown that PF68 has multiple effects in CHO cell culture. This includes the enhancement of growth under agitated culture mode and reduced adherence in stationary culture. The PF68 coating the membrane serves to enhance robustness, reduce surface attachment and limit the damage of cells exposed to high shear forces or proteolytic enzymes.

Chapter 10

10. The effect of PF68 on Protein Production and Glycosylation

10.1. Introduction

The media components added to cell culture may be monitored for inhibitory effects on cell growth or protein production. In the case of our studies, the addition of PF68 was monitored for its effects on cell adherence (Chapter 8) and benefits on protection of cells from high levels of shear and proteolytic damage (Chapter 9). The benefits of adding PF68 to cell culture could be nullified if there is an inhibitory effect on the recombinant protein production of IFN-beta and tPA in cell culture.

In this chapter we focus on the effects of PF68 on the recombinant protein production of IFN-beta and tPA produced in CHO cells. The effect of PF68 on protein production is compared using different modes of culture (T-flasks, spinners and bioreactors). Also, the effects of PF68 and shear stress on the heterogeneity of recombinant human interferon-beta were investigated.

10.2. Results

10.2.1. PF68 and Protein Production in T-flask cultures

The removal of PF68 affects the growth of cells in stationary T-flasks; as the percentage of PF68 decreases, the cell adherence increases (explained in Chapter 8). The effect of PF68 on recombinant protein production was tested in adherent cultures using CHO cells producing IFN-beta.

Cell concentrations remain relatively constant between $3.41x10^6$ cells/mL to $4.08x10^6$ cells/mL after 4 days of growth (Figure 10.1A). However, the IFN-beta producing cell line showed a slight but significant increase in recombinant protein production with the increase in PF68 in the media. Figure 10.1B shows a slight trend of product increase as PF68 concentrations increase, as indicated by the equations: native $y=6.09x10^6(x)+0.96x10^6$ ($r^2 = 0.98$) and denatured $y=8.93x10^6(x)+1.04x10^6$ ($r^2 = 0.98$). Both the native and denatured protein products show a slight upward trend of protein yield. This leads us to believe that PF68 concentrations have a positive effect on protein production in CHO cells producing IFN-beta.

The product titres of tPA from T-flask cultures of CHO cells are visualized in Figures 10.2 and 10.3. There appeared to be no increase in tPA produced when PF68 concentrations increased (p-value = 0.8). Cells grown in 0 % PF68 yielded a total maximum cell count of $4.04x10^6$ cells/mL, where the product yield was 3000 IU/mL. At the varying concentrations of PF68 (0.01 % to 0.5 %), the cell counts varied from $4.0x10^6$ cells/mL to $4.6x10^6$ cells/mL, but product yield was between 2700 IU/mL and 3500 IU/mL, thus indicating that there is no impact of PF68 concentrations on the production of tPA.

10.2.2. PF68 and ELISA

The presence of PF68 could interfere with the ELISA assay, thus giving an erroneous protein measurement. Thus to eliminate the possibility of an interference, the ELISA assay was performed on samples in the presence and absence of PF68. The results are shown in Figure 10.4. Samples of tPA in PF68 containing and PF68-free media were

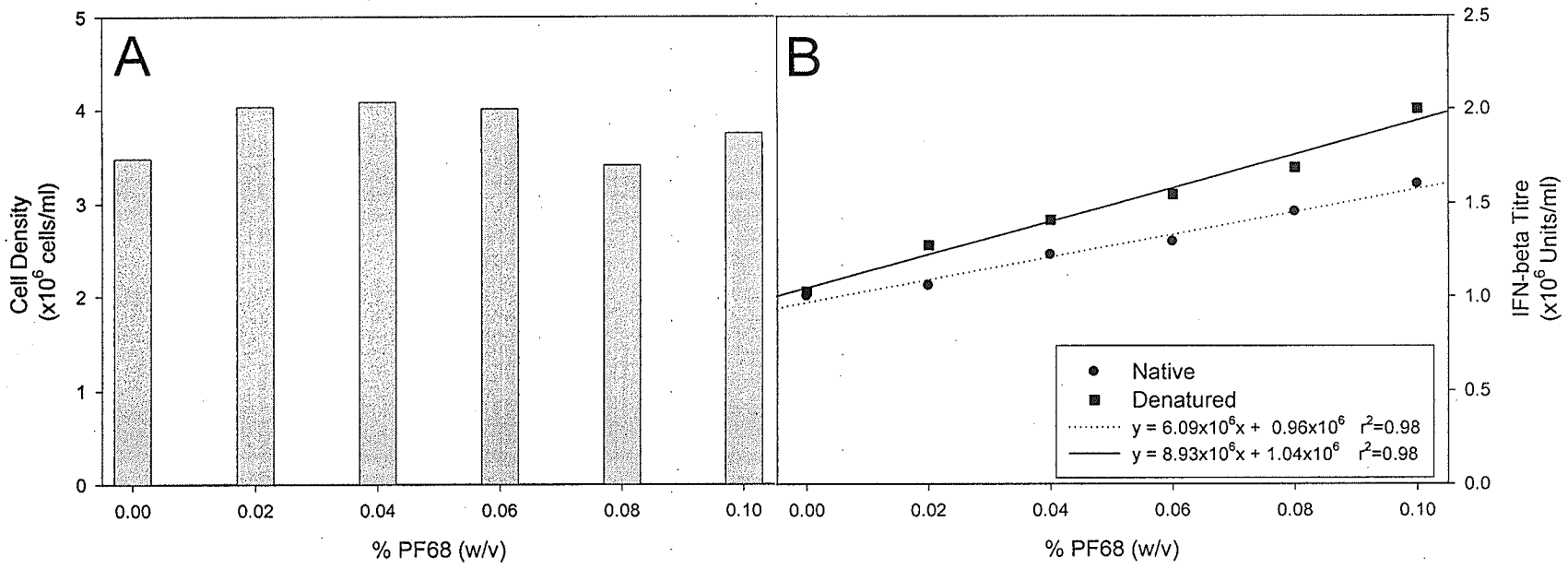


Figure 10.1: Cell growth (A) and IFN-beta titre (B) from CHO cells grown in stationary T-flasks for 4 days under different concentrations of PF68. The cells were enumerated in trypan blue while product yields were calculated by ELISA.

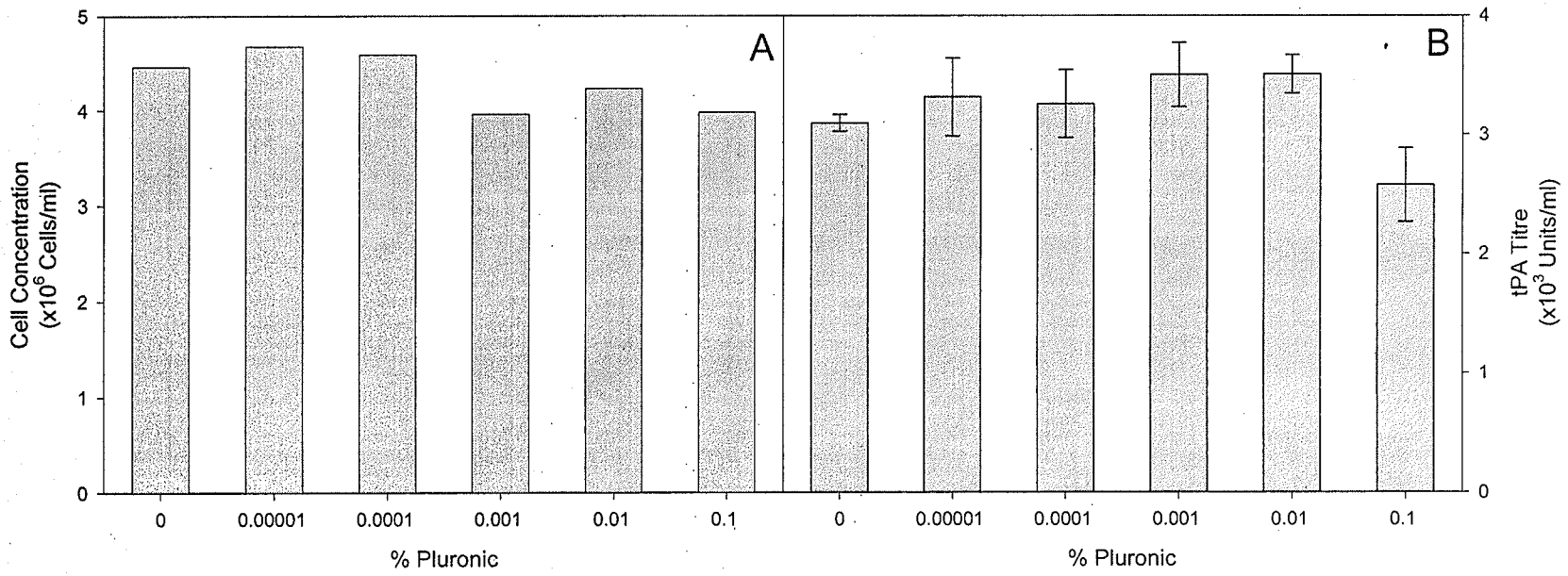


Figure 10.2: Cell growth (A) and tPA titre (B) from cells grown in stationary T-flasks under different concentrations of PF68. The cells were enumerated in trypan blue while product yield was calculated by ELISA.

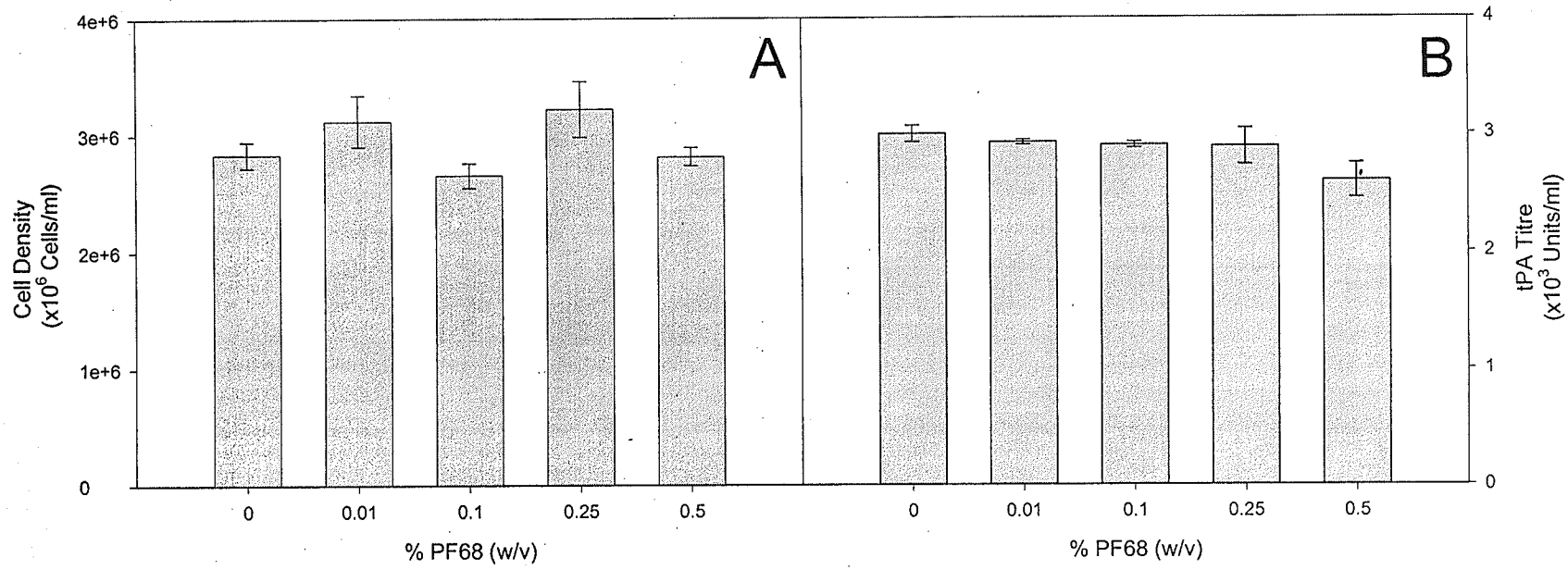


Figure 10.3: Cell growth (A) and tPA titre (B) from cells grown in stationary T-flasks under different concentrations of PF68. The cells were enumerated in trypan blue while product yield was calculated by ELISA. The cell densities and titres are as determined from duplicate samples, with error represented as SEM.

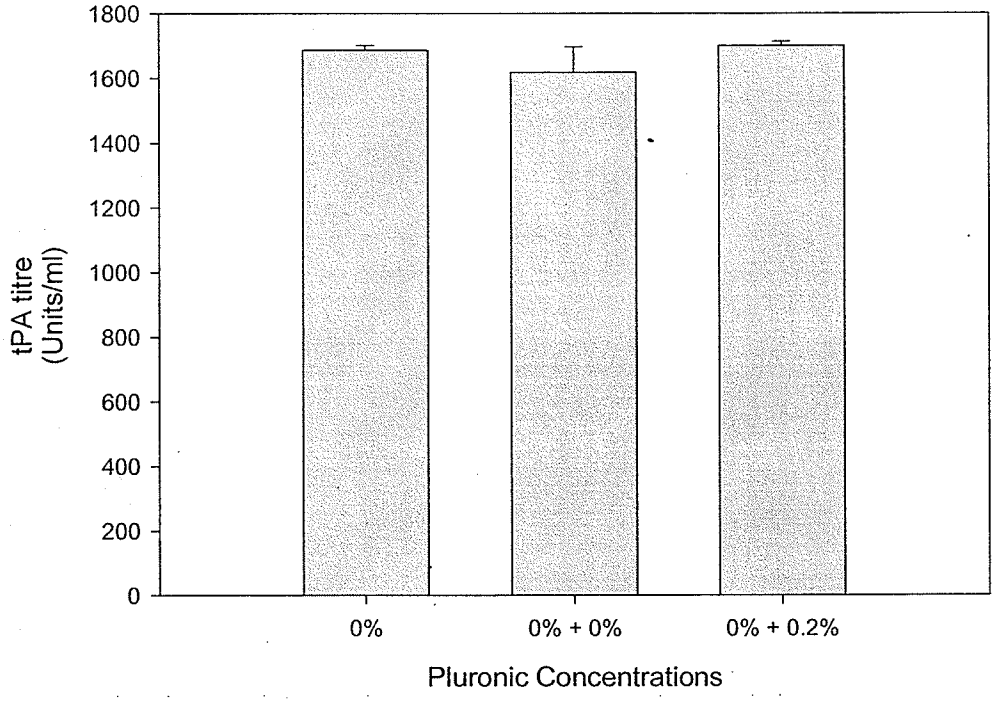


Figure 10.4: The effect of PF68 on the determination of tPA titres by ELISA. The tPA supernatant samples tested were from T-flask cultures with cells in PF68-free media (0 %), PF68-free grown + diluted with PF68-free media (0 % + 0 %), and PF68-free grown and diluted with 0.2 % PF68 containing media resulting in 0.1 % PF68 within the tested sample (0 % + 0.2 %). The titres are as determined from duplicate samples, with error represented as SEM.

assayed by ELISA after dilution with media containing PF68 or no PF68. The original samples obtained from cultures were from 0 % media samples which were assayed to contain 1700 Units/mL tPA. A dilution of the sample obtained from 0 % PF68 with 0 % PF68 media resulted in a titre of 1620±80 Units/mL whereas diluting the same sample such that the final concentration of the sample was 0.1 % resulted in a measured titre of 1700±13 Units/mL. Thus it was concluded that the addition of PF68 does not cause a false increase in the samples being assayed by ELISAs.

10.2.3. PF68 and protein production in spinner cultures

Spinner cultures with CHO cells producing IFN-beta showed that the production of IFN-beta was not dependent on the presence of PF68. In Figure 10.5, there is an increase in product titres when cells were grown in the absence of PF68. The product yields are similar until day 4, where a maximum yield of about 2.80×10^6 units/mL were present in the culture. The samples for glycan analysis from the spinner cultures were obtained at day 4, as the cell yield and product titre between both cultures were consistent.

Analysis of the tPA producing cell line showed that cells removed from PF68 for 3 passages and then inoculated into spinner flasks showed a similar growth pattern as that described above (Figure 10.6). Where a maximum cell yield reached was at day 5, with 3.13×10^6 cells/mL in the presence of PF68, a maximum of only 0.85×10^6 cells/mL was reached at day 6 in the absence of PF68. Protein production analysis by tPA ELISA shows that cells grown in the absence of PF68 had a maximum titre of 3160 units/mL compared to a maximum titre of 6374 units/mL from cells grown in the presence of

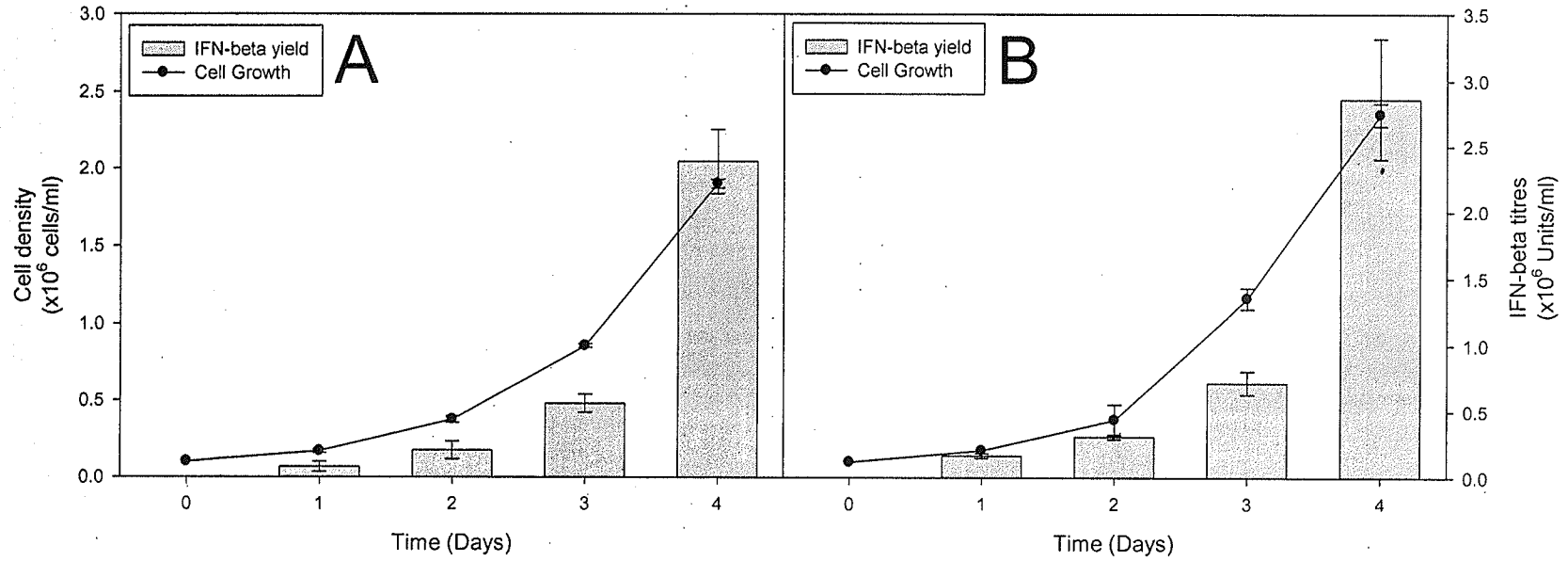


Figure 10.5: Growth and Productivity of CHO cells producing IFN-beta grown in 100mL Spinner flasks. The cells were grown in 0 % for 3 passages before being inoculated into the spinners. The left panel (A) represents the growth and productivity of cells grown in 0 % PF68, and the right panel (B) represents the growth and productivity of cells grown in 0.1 % PF68. The productivity was assayed by an ELISA assay. The titres are as determined from duplicate samples, with error represented as SEM.

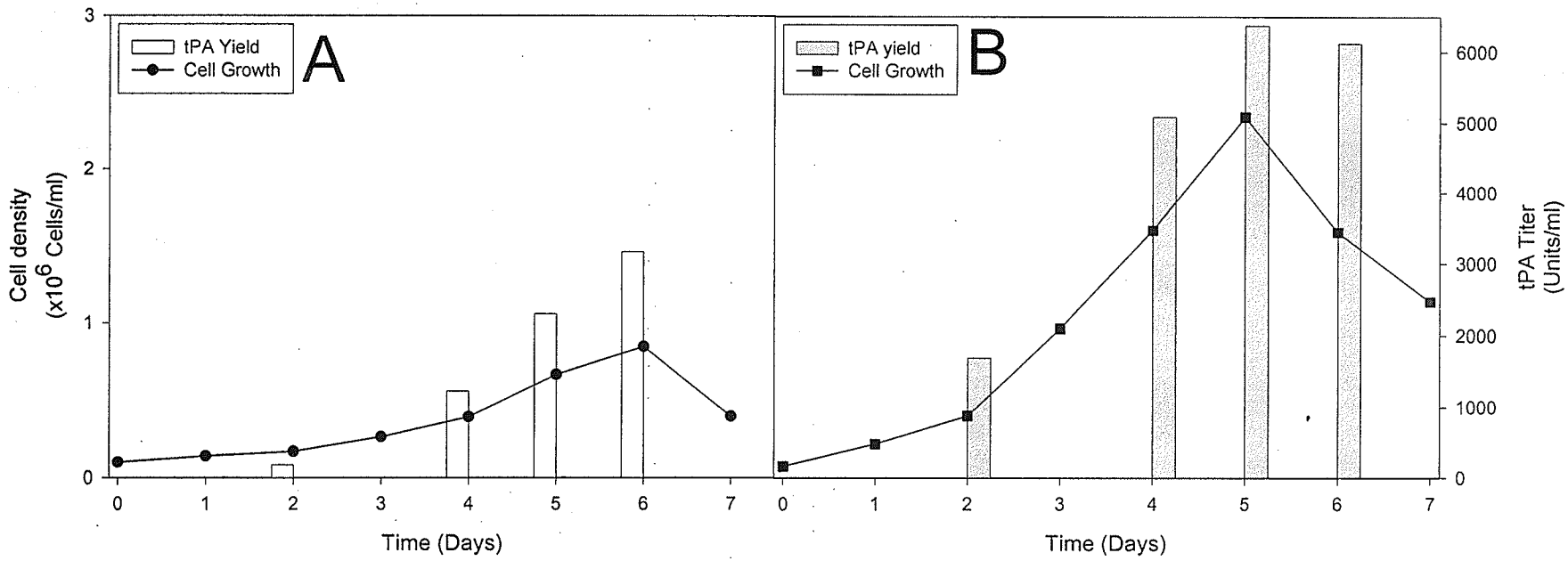


Figure 10.6: Growth and Productivity of CHO cells producing tPA grown in 100mL Spinner flasks. The cells were grown in 0 % for 3 passages before being inoculated into the spinners. The left panel (A) represents the growth and productivity of cells grown in 0 % PF68, and the right panel (B) represents the growth and productivity of cells grown in 0.1 % PF68. The tPA titre was determined by an ELISA assay. The cell density and tPA titres are from representative cultures.

PF68. Although there was a difference in the volumetric product of tPA from cells grown in the presence and absence of PF68, this appeared to be due to cell growth. The calculated cell specific productivity for cells grown in both PF68-free and in the presence of PF68 was not significantly different (1.5 mUnits/cell/day). Thus PF68 did not have any effect on the protein productivity of the tPA producing cell line.

10.2.4. PF68 and Protein Production in bioreactor cultures

The IFN-beta produced from the bioreactor cultures appears to be minimal in the absence of PF68, as visualized in Figure 10.7. This is due to the killing of cells in the absence of PF68 by shear forces. The maximum product yield obtained in the presence of PF68 is $3.5x10^6$ units/mL denatured product yield.

10.2.5. The effect of PF68 on the glycosylation of IFN-beta.

It is well documented that PF68 protects cells from the shear forces. However, very little has been reported about the effect of PF68 on the glycosylation of the protein. We chose to use spinner flasks cultures to study the effects of PF68 on the glycosylation of IFN-beta because significant growth could be obtained even in the absence of PF68.

Analysis of the IFN-beta produced by cells grown in the presence and absence of PF68 in spinner cultures showed that there was no effect of PF68 on the glycosylation. The glycan structures of IFN-beta are shown in Chapter 7. Glycan analysis (Figure 10.8) shows that there is similar glycosylation of the products from 0 % PF68 spinners and 0.1 % PF68 spinners with rotation speed of 45 rpm throughout the culture period. The integration of the area under the peaks (Figure 10.9) shows that 50 % of the glycans are

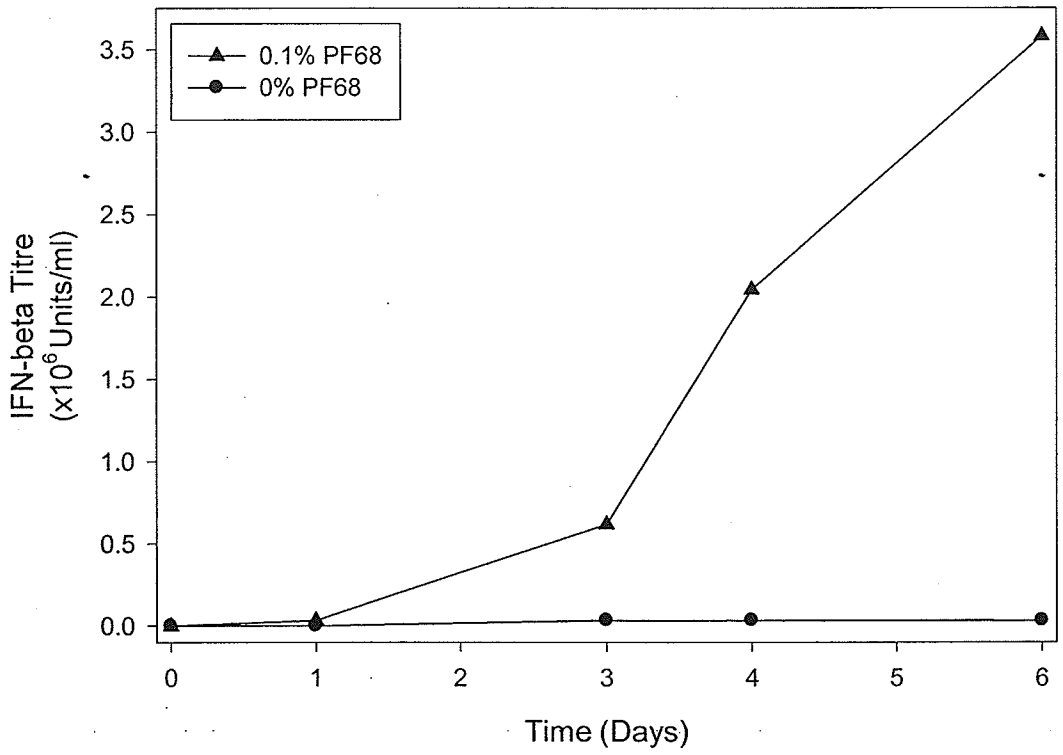


Figure 10.7: The IFN-beta produced from a CHO cells in a 2L bioreactor culture (Figure 9.3) in PF68-free and 0.1 % PF68 containing media. IFN-beta titres were assayed by ELISA.

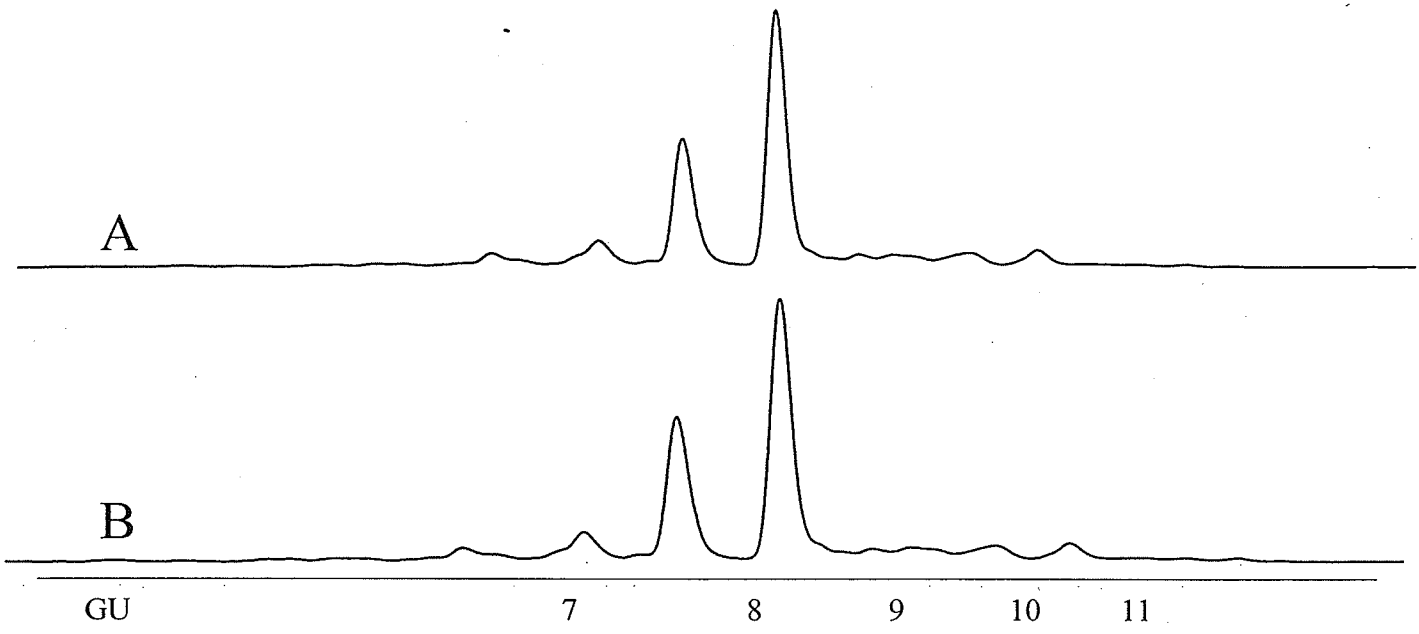


Figure 10.8: Representative glycan profiles from IFN-beta samples from PF68-free (A) and PF68 (B) spinners. The spinners had a stir rate of 45 rpm throughout the culture period.

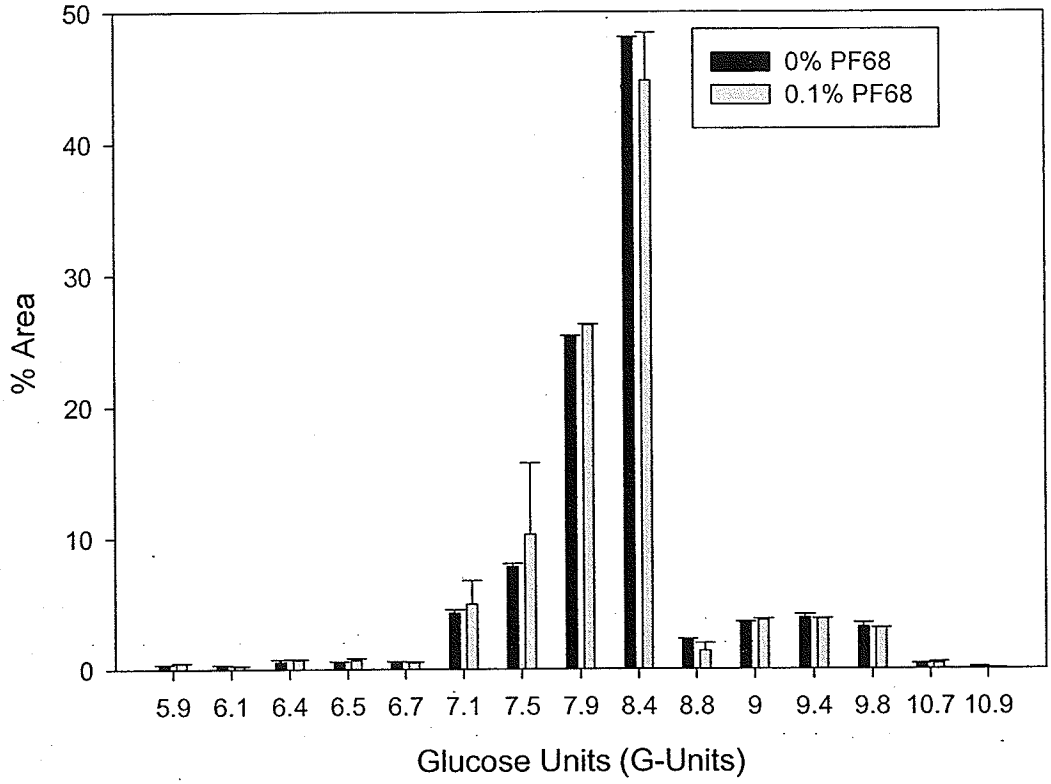


Figure 10.9: Integration analysis from Glycan analysis profiles from PF68-free and PF68 containing IFN-beta glycans from the profiles in Figure 10.8. The glycan structures are as identified in Chapter 7 (Section 7.2.2). The integration values are expressed as a mean (±SEM) where (n=2).

of the bisialylated biantennary glycan form, 20 % of the glycans are of the monosialylated biantennary glycan form, 10 % are of the monosialylated form, and roughly 20-30 % are of triantennary and tetraantennary moieties.

10.2.6. The effect of shear forces on the glycosylation of IFN-beta

Although there is no effect of PF68 on glycan structures, there was a difference in the glycan profile of IFN-beta from spinners at a high (100 rpm) (Figure 10.10) or low (45 rpm) (Figure 10.8) speeds of rotation in the presence and absence of PF68. Figure 10.11 shows the integration of the area under the peaks from the cultures agitated at 100 rpm. The integration shows that 50 % of the glycans are of the A2G2FS2 moiety, 18 % of the glycans are of the A2G2FS1 moiety, 10 % are of the A2G2FS0 moiety, and the majority of the remaining glycans are triantennary or tetrantennary glycans.

Figure 10.12 shows the integrated areas from IFN-beta glycans isolated from all four culture conditions. The increased speed of rotation at 100 rpm appears to decrease the monosialylated biantennary structure (7.9 GU) of IFN-beta by 8 %, where the 45 rpm cultures had a monosialylated peak with over 25 % conformity and the glycans from the 100 rpm cultures had just 18 % (Figure 10.11). This decrease was present for glycans formed in the presence or absence of PF68. This decrease in the monosialylated biantennary structure was offset by an increase in the bisialylated biantennary and trisialylated triantennary glycans structures.

The glycan sialylation can also be detected by WAX (Weak Anion Exchange) as the sialic acids give the glycan an overall negative charge, thus can be separated out by

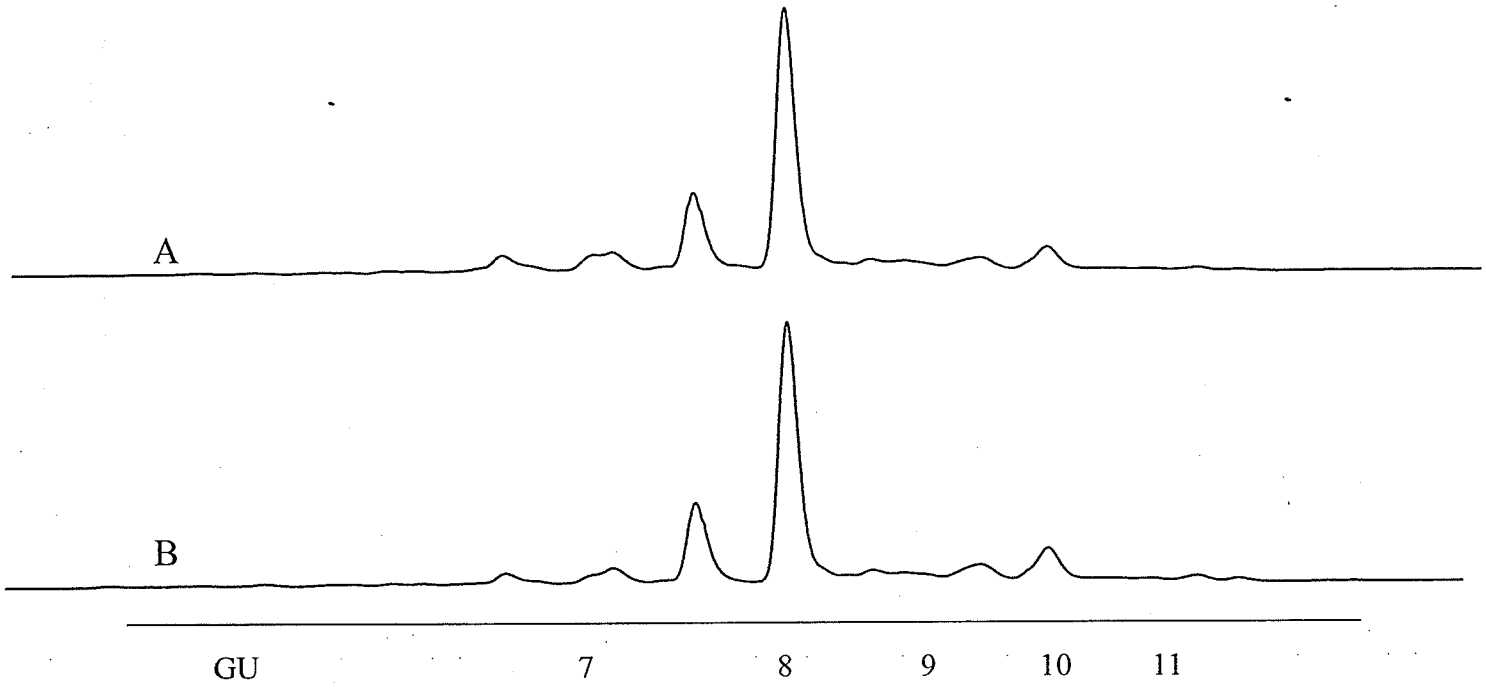


Figure 10.10: Glycan analysis profiles from PF68-free (A) and PF68 containing (B) IFN-beta glycans grown in 100 rpm spinners.

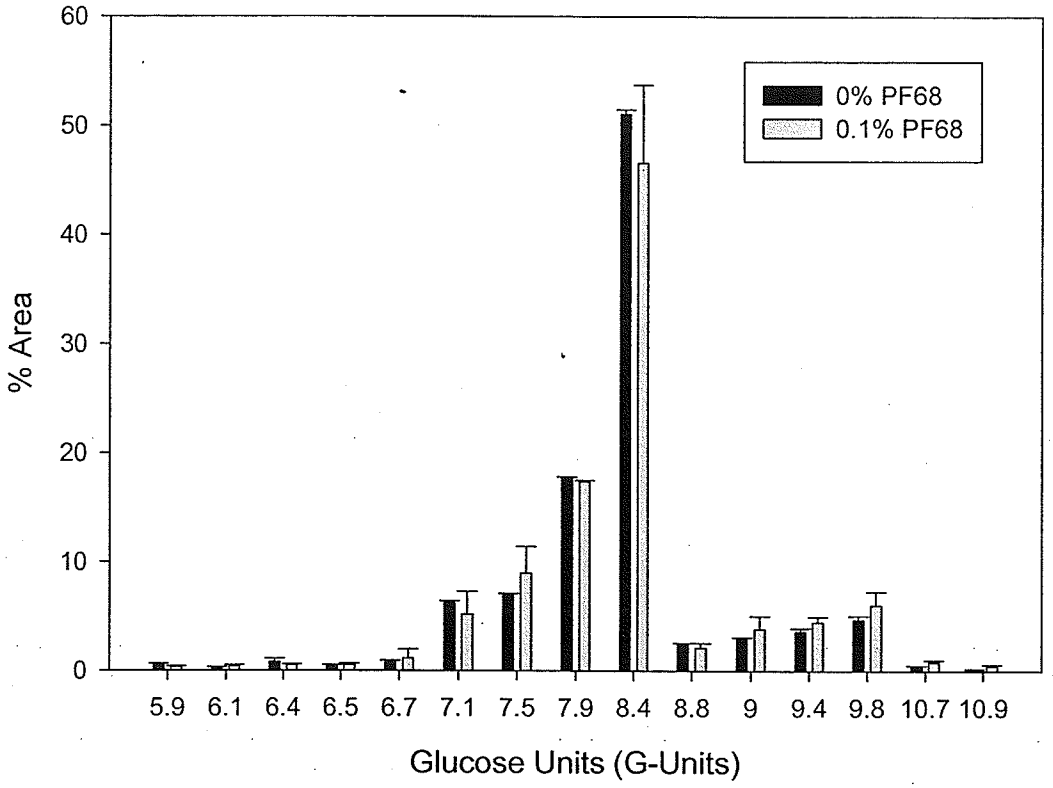


Figure 10.11: Integration analysis from Glycan analysis profiles (as shown in Figure 10.10) from PF68-free and PF68 containing IFN-beta glycans. The integrated values are expressed as a mean (±SEM) where (n=2).

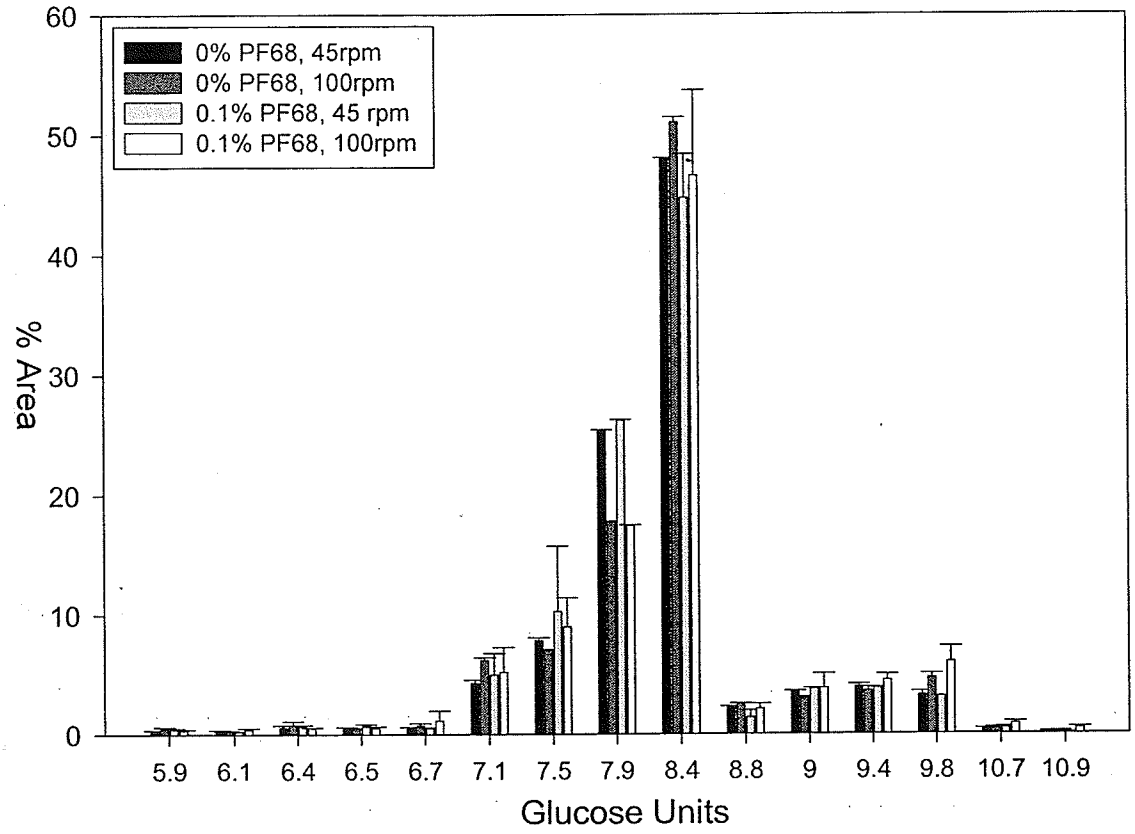


Figure 10.12: The effect of PF68 and stir speeds on the glycosylation of IFN-beta. The cells were inoculated into 100 mL spinner flasks with PF68-free or PF68 containing media and grown for 5 days. The media was filtered then frozen at -20°C until purification and sample preparation for glycan analysis. The peaks obtained in the 2-AB labeled N-glycan extraction from IFN-beta were compared to a dextran ladder to obtain Glucose Unit values. The percent area was calculated from integration of area under the peaks obtained. The values are expressed as a mean (±SEM) where (n=2).

charge. The degree of sialylation in WAX correlates with retention time with the more charged glycans (tetrasialylated) retained for longer in the column. Figure 10.13 shows the results from the elution of the charged glycans from the WAX column, where the non-sialylated glycans are minimal, and the majority of the glycans are of the A2G2FS1 and A2G2FS2 forms. The A2G2FS1 glycan shows a slight difference between the higher spinner stir rates compared to the 45 rpm spinners, where overall sialylation is decreased at the lower rotation speed of 45 rpm (Figure 10.14) by 5 % or more. However, as shown with the NP-HPLC results there are no differences between the glycans isolated from PF68 containing and PF68-free media.

10.3. Discussion:

The addition of PF68 appeared to affect the protein production of IFN-beta and not tPA. Thus the effect of PF68 addition on protein production appears to be cell line dependent. The addition of PF68 at low concentrations (between 0 % and 0.1 %) increased production of IFN-beta by up to 2-fold (denatured IFN-beta) compared to samples produced in the absence of PF68. There was no effect from the addition of PF68 on the production by the tPA cell line. The addition of PF68 does not affect cell growth at these concentrations; however PF68 addition at these concentrations decreased cell to surface adhesion in stationary T-flasks, where the adherence of the cells decreased from 60 % in the absence of PF68 to 5 % in the presence of 0.1 % PF68 (Chapter 8).

A proposed explanation for the higher production rate is that cells in lower concentrations of PF68 devote more of their protein production towards adhesion

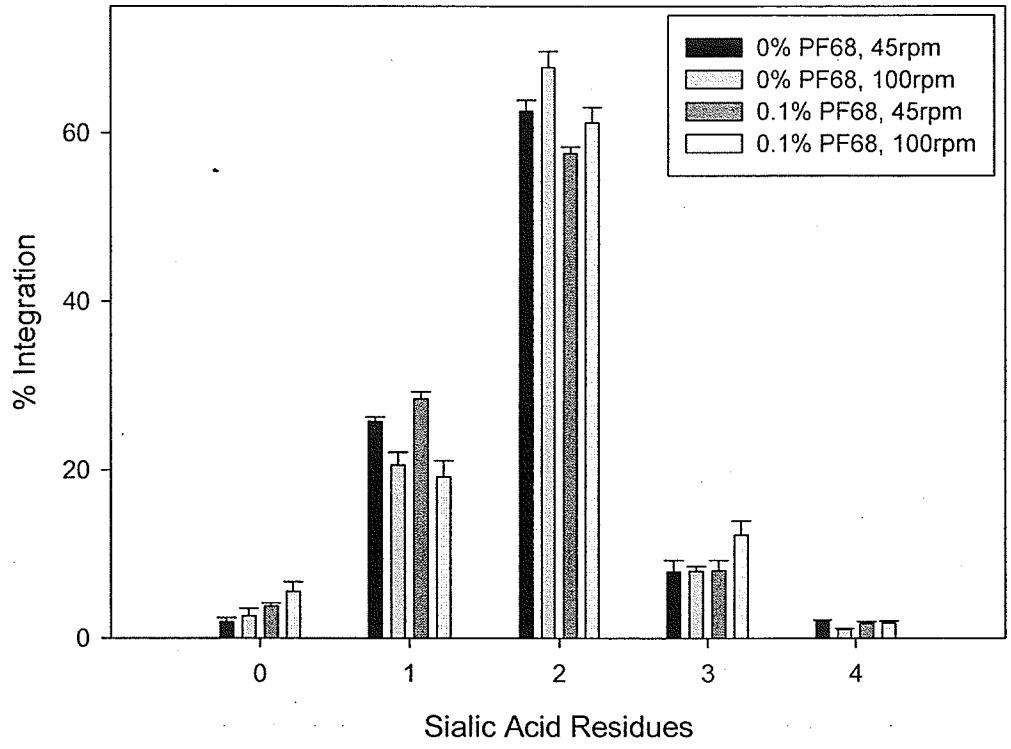


Figure 10.13: Sialic acid content from glycans isolated from spinner flasks containing 0 % PF68 and 0.1 % PF68 with spinner speeds of 45 rpm and 100 rpm. The sialic acid was detected by WAX – HPLC. The sialic acid residues refer to the sialic acids present on each glycan isolated from IFN-beta. The values are expressed as a mean (±SEM) where (n=2).

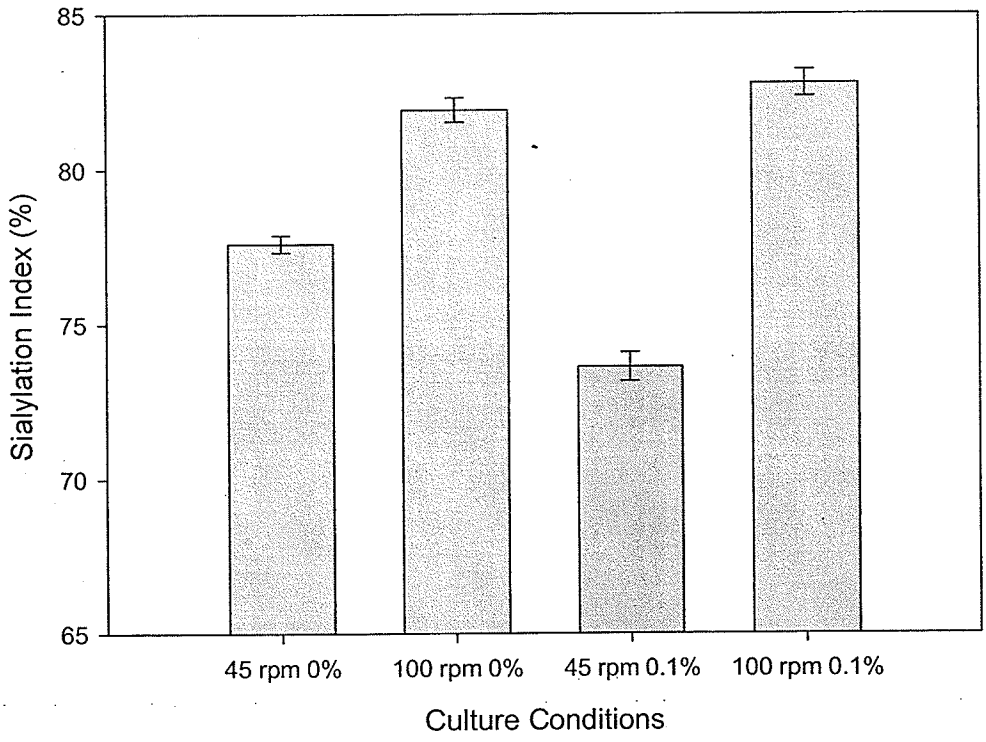


Figure 10.14: Sialylation index of glycans isolated from spinner flasks containing 0 % PF68 and 0.1 % PF68 with spinner speeds of 45 rpm and 100 rpm. The sialylation index is calculated as shown in Section 2.6.6. The values are expressed as a mean (±SEM) where (n=2).

proteins compared to cells growing in suspension. Where adhesion proteins are not necessary for growth in suspension cell culture processes, the energy could be diverted to recombinant protein formation. The overexpression of αCaMKII (Ca^{2+} / calmodulin – dependent protein kinase) to increase cell to surface adhesion caused transfected cells grew 50 % slower than nontransfected cells (Masse and Kelly 1997).

The production of IFN-beta was not greatly effected in the presence of PF68 in the spinner cultures. However, the addition of PF68 in the case of the tPA producing cell line impacted the cell resistance to shear, and thus cell concentrations were maintained in the presence of PF68. Therefore the volumetric protein formation was enhanced from the tPA producing cell line in the presence of PF68. The specific product formation was not impacted greatly where the presence of PF68 had a calculated specific productivity of 1.486 mUnits/cell-day compared to 1.537 mUnits/cell-day in the absence of PF68. A similar effect was seen in the 2L bioreactor culture, where the cell yield of CHO cells producing IFN-beta, impacted the protein yields obtained from these cultures. Thus in the absence of PF68 the protein yields remained close to nil compared to the 0.1 % PF68 containing bioreactor culture.

The enhancement of protein yield is generally due to an increase in cell yields due to the protection of cells in a high shear environment. The cell yields are maintained, and in some cases are enhanced, thus leading to an increase in the protein titres due to a greater population of cells with a similar cell specific productivity. Sakai *et al.* (2002) showed that there was no growth promoting activity when PF68 or Tween 80 was added into

culture, and production levels of human IFN-gamma were similar. Similar growth was reported when growing non-transfected CHO cells (Sakai *et al.*, 2001).

The addition of different media components in cell cultures has often been found to affect the recombinant protein production from these cells. The effect of PF68 on recombinant protein production has been described to be cell line specific. The effect of PF68 on CHO cells producing human growth hormone (hGH) was shown when cells that were exposed to shear stress ceased hGH production; however addition of 0.2 % PF68 allowed continued production of hGH (Keane *et al.*, 2003).

Apart from mammalian cell culture, PF68 is also used for the culturing of other cell lines such as insect and plant cells. The addition of PF68 has been reported to increase the production of mGM-CSF from *Nicotiana tabacum* cells (Lee and Kim, 2002). PF68 was shown to stimulate the anthroquinone product release from plant cells. The use of PF68 at 2 % (w/v) improves the intracellular release of secondary metabolites from *Morinda citrifotia* cells (Bassetti *et al.*, 1995). The use of PF68 for the growth and production of recombinant proteins in Sf-9 insect cells, where the presence of PF68 increased the maximum recombinant protein concentration, yield, productivity and specific production rate by 10-fold in cultures supplemented with PF68 (Palomares *et al.*, 2000).

In most cases the absence of PF68 has been shown to negatively affect the growth of cells, and thus negatively affect the production of recombinant proteins. Castro *et al.* (1995) reported that the addition of PF68 stimulated CHO cell growth although the expression of human interferon-gamma was not improved. Ghebeh *et al.* (2002) reported

that although the cells were able to grow under high levels of shear stress in the presence of PF68, there was no effect on the production of EPO from CHO cells. The EPO produced (348 +/- 40 units/mL) was consistent across the cultures.

The effect of PF68 on protein glycosylation was also tested in this study. Recombinant IFN-beta was isolated from supernatants from spinner cultures and tested for differences in glycosylation. As mentioned in Chapter 7, IFN-beta has 1 glycosylation site, which varies in the microheterogeneity depending on culture parameters. The overall yield of protein was relatively similar when comparing the PF68-free and PF68-containing samples, and thus the IFN-beta was purified and tested for differences in glycosylation.

There appeared to be no differences in the macroheterogeneity when comparing the IFN-beta produced in PF68-free or PF68-containing culture media ($p=0.18$). As well, there was no significant difference when comparing the glycosylated and non-glycosylated protein yields at a higher spinner rotation speed compared to the control 45 rpm ($p=0.90$). This finding was different than that by Keane *et al.* (2003) where the production of type II tPA (with 2 N-glycosylation sites) was 74 % in the presence of high shear stresses compared to type I tPA (3 N-glycosylation sites) with 26 %. Thus, the quality of the protein produced was lowered under high shear conditions.

In this study the glycosylation of the IFN-beta was found to be affected by the high rotation speeds of the spinner culture. The differences in glycosylation of the IFN-beta were found to differ from the 45 rpm culture in the quantity of monosialylated A2G2F glycans. IFN-beta isolated from the CHO cell cultures has predominantly a biantennary

fucosylated monosialylated and biantennary fucosylated di-sialylated glycan. The predominant form isolated from cultures is of the A2G2FS2 form. However, under shear stress exposure the quantity of monosialylated glycan forms is decreased, while the di-sialylated moiety remains constant at roughly 50 %.

The sialylation of beta-interferon was decreased by 6-7 % under the condition of high shear. This result is consistent with those of Joosten and Shuler (2003) where they reported that the highest level of sialylation (9 %) of recombinant secreted alkaline phosphatase (SEAP) from the *Trichoplusia ni* insect cell line, Tn-4s was obtained in the culture with the lowest agitation rate. Furthermore the lowest level of sialylation (2.6 %) was obtained in the 150 rpm culture and suggests a detrimental effect of shear on glycosylation. Although the addition of PF68 did not affect the glycosylation of the SEAP.

The comparison of glycans from IFN-beta produced in CHO cells in the presence and absence of PF68 showed that this surfactant had no effect on the glycosylation of the protein. However, a difference was seen when comparing the glycans isolated from proteins produced at a higher shear rate than the control cultures. There was a decrease in the monosialylated glycan compared to the control spinner cultures with rotation speed of 45 rpm.

Section B - Discussion

The use of PF68 in mammalian cell culture is wide spread, as it has been shown previously to protect cells from high shear forces caused by air sparging as well as by rotation of the impellor. The use of PF68 has been reported to prevent cell to bubble attachment, and thus death by hydrodynamic damaging forces due to the collapsing of bubbles. In this section we show that PF68 protects cells from high shear forces caused by spinner speeds of 500 rpm, as well as shaker flask with high rotation speeds of 300 rpm, both of which allow for bubble formation. It is also evident that PF68 protects cells from high shear forces in a bubble free environment, as that caused by high rotation speeds in a viscometer. We show that PF68 does not have a long term protection and thus is only protective when present during high shear force exposure.

The experiments done here show a mechanical effect of the PF68 on the cells. In the presence of PF68, exposing the cells to shear forces induce minimal disruption on the cells. However, the protective effect of PF68 can be inferred to be also due to a biological effect. The removal of PF68 from stationary T-flask cultures induced the attachment of cells to the T-flask. Although the addition of varying concentrations of PF68 did not affect cell growth, the recombinant protein production varied, showing that the effect of PF68 on recombinant protein production is cell line dependent.

The treatment of cells to trypsin caused proteolytic damage to the cells, and thus led to lower cell viability in cultures not exposed to PF68. The exposure of cells with trypsinization to PF68 led to a lower percentage, of cells being affected by the trypsin. As well, cell counting after trypsin treatment was slightly improved (10%) upon addition of

PF68 to the cells prior to cell density estimation using the trypan blue exclusion method. The addition of PF68 to cells treated to trypsin for up to 2 hours then inoculated into spinner flasks with and without PF68 induced 2-fold greater cell growth in PF68 containing medium. Thus, the PF68 works to protect weak cells, perhaps by plugging holes induced in the membrane by either shear forces or by proteolytic damage.

Although Palomares *et al.* (2000) speculate that PF68 is taken up into cells due to the long term protection of cells after PF68 has been removed from culture media, we fail to see this long term protection in our shear experiments. In our studies, once PF68 has been removed from culture media, cells were found to be affected by the shear forces (Section 9.2.5). However, in the adherence experiments, there appeared to be a gradual increase in cell adherence over passages where PF68 was removed (Section 8.2.3). The protection of cells from high shear forces and proteolytic damage occured in the presence of PF68.

The addition of PF68 in the media is important to ensure the protection of the cells from high shear forces. It is important for maximization of the recombinant protein production to scale up cultures in bioreactors; in cultures with PF68 we were able to attain higher growth and production, compared to PF68-free media. Therefore, the use of PF68 for the growth of CHO cells producing the recombinant proteins is essential to maximize cell growth and recombinant protein production from larger scale bioreactor cultures.

Chapter 11

11. General Discussion

In this study, I evaluated the effects of using PF68 and microcarriers on the growth of cells, as well as their effects on productivity and glycosylation of the recombinant proteins produced. Mammalian cells are prone to shear force damage, and thus media additives, such as PF68, must be incorporated to help with the protection of the cells. PF68 has been used in media compositions for numerous years; however the mechanism of protection that PF68 offers is still not properly elucidated. The two main theories are based on mechanical effects, whereby cells are coated and more protected, and biological effects whereby PF68 is incorporated into the cellular membrane.

The addition of PF68 allows protection of cells from high shear forces, and also allows for the decrease of attachment of cells in static cultures (Tharmalingam *et al.*, 2008a). The analysis of glycosylation profiles of protein produced in the presence or absence of PF68 showed that there is no effect on glycosylation. However, the introduction of shear stresses caused the decrease of sialylation giving a higher percentage of the A2G2FS1 compared to the A2G2FS2 (See Section 7.2.2).

Macroporous microcarriers such as Cytopore entrap cells in a mesh network allowing growth to high cell concentrations in a protected environment from high shear forces within a pseudo-suspension stirred culture. In this study we determined the relationship between the cell density and the specific productivity of Chinese hamster ovary (CHO) cells in both Cytopore 1 and 2 microcarriers. Cells producing recombinant IFN-beta within a Cytopore 2 microcarrier culture showed a 5-fold increase in the specific

productivity and a 3-fold increase in the volumetric product titre compared to cells grown in an equivalent suspension culture. The densities reached within the microcarriers were $2x10^8$ cells/cm^3, which is comparable to the cell density in typical animal tissue (Palsson and Bhatia, 2003). This high density seems to account for the observed increase in specific productivity of recombinant proteins in these microcarrier systems.

An optimal biphasic temperature-shift regime was applied to Cytopore 2 microcarrier cultures in an attempt to further enhance the productivity of IFN-beta. Under these conditions the volumetric productivity of IFN-beta was enhanced 4.2-fold compared to a single temperature suspension culture in a controlled bench-top bioreactor. Furthermore the degree of molecular aggregation was reduced significantly, largely due to the lower temperature but also partially due to the presence of microcarriers. These results indicate that the hypothermic conditions in a Cytopore culture had a combined and possibly synergistic effect of increasing volumetric productivity of the recombinant protein.

The major achievement of this work was the enhancement of the volumetric production of IFN-beta and tPA by over 2-fold, and the specific productivity enhancement of these proteins (up to 5-fold) in Cytopore 2 microcarriers (Tharmalingam *et al.*, 2008b). Furthermore, there was a 5-fold enhancement when combined with hypothermic culture conditions compared to a suspension bioreactor culture (Tharmalingam *et al.*, 2008c). The approach that we undertook to increase overall product titres was by obtaining high specific productivities at high cell densities. Generally, due to costs of production, the use of microcarriers is not adopted for large

scale production of recombinant proteins. Most approaches focus on obtaining cells adapted to suspension cultures, and thus by using macroporous microcarriers we have shown an alternative approach to increasing recombinant protein production.

The overall glycan structure distribution was similar to the suspension cultures (biantennary fucosylated mono- and di-sialylated glycans) with a higher percentage of the di-sialylated forms (40-50 %). However with the combination of Cytopore and temperature shifts, there was a higher quantity of glycosylated protein, but a shift towards lower sialylated glycans (A2G2FS0 and A2G2FS1) compared to the suspension cultures.

Although the use of PF68 and microcarriers can be beneficial for cell growth and protein production, it is important to ensure consistent product quality, in particular the glycosylation. The glycosylation of the recombinant protein ensures protein efficacy and low immunogenicity, and thus the protein must be analyzed thoroughly.

11.1. Future Work

11.1.1. PF68

The use of PF68 in serum free media is important to allow for cells to be protected from high shear forces in spinners and bioreactors, as well as for the inhibition of adhesion to T-flasks. Although the analysis that we completed here showed no differences in glycosylation due to PF68, I did show that the glycosylation is altered when spinner rotation speeds are increased. Thus further work is required to show whether this difference in glycosylation is due to the up regulation of enzymes (eg. sialyltransferase) when exposed to higher oxygen transfer from the higher stir rates. This

can be attempted by isolating proteins from the golgi that may be differentially expressed in high and low oxygen conditions then using mass spectrometry to identify the protein. It would be also interesting to see whether the differences in glycosylation are due to the exposure of glycans to high shear stresses, and not due to the differences in production. This can be attempted by exposing sialylated standards to high shear stresses then comparing the microheterogeneity to the original standards.

Another aspect that must be regarded is the recombinant product formation enhancement. In my study I reported an increase in product formation from the IFN-beta cell line and not the tPA producing cell line; thus it would be informative to look at the genetic level to determine what would cause the differences in product formation in the presence of PF68. Also it would be interesting to analyze the differences in the proteomes that would lead to increased adhesion to T-flask surfaces in the absence of PF68.

11.1.2. Microcarriers

For further work to be done on microcarriers, I believe that it would be beneficial to scale the culture levels higher for further analysis. Our lab has been involved in successfully developing perfusion modes of culture in 2L bioreactors for human IFN-beta production (Rodriguez et al, 2008). We have reported that IFN-beta aggregation could be decreased to less than 10% while increasing productivity ($77x10^6$ units/ml). It would be interesting to see if the incorporation of Cytopore in a perfusion system could increase protein yields further by decreasing aggregation.

Another interesting aspect that should be further explored, is the reason for the increased specific productivity when the cell density reaches high concentrations. In particular, we could investigate the possibility of a decrease in protease formation in high cell density cultures or the up regulation of genes that would lead to this increase. The information obtained from such analysis could be used to genetically modify cell lines, such that the cells could produce the recombinant protein at a high specific productivity without the use of Cytopore microcarriers, thus being more cost effective.

References

Ahmed F, Alexandridis P, Neelamegham S. 2001. Synthesis and application of fluorescein-labeled Pluronic block copolymers to the study of polymer-surface interactions. Langmuir 17:537-546.

Al-Rubeai M, Emery AN, Chalder S. 1992. The effect of Pluronic F-68 on hybridoma cells in continuous culture. Appl. Microbiol. Biotechnol. 37:44-45.

Al-Rubeai M, Emery AN, Chaler S, Goldman MH. 1993. A flow cytometric study of hydrodynamic damage to mammalian cells. J. Biotechnol. 31:161-177.

Al-Rubeai M, Singh RP, Emery AN, Zhang Z. 1995. Cell cycle and cell size dependence of susceptibility of hydrodynamic forces. Biotechnol. Bioeng. 46:88-92.

Altamirano C, Paredes C, Cairo JJ, Godia F. 2000. Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. Biotechnol. Progr. 16(1):69-75.

Andersen DC, Bridges T, Gawlitzek M, Hoy C. 2000. Multiple cell culture factors can affect the glycosylation of Asn-184 in CHO-produced tissue-type plasminogen activator. Biotechnol. Bioeng. 70(1):25-31.

Aquino DA. 1984. Immunocytochemical localization of a chondroitin sulfate proteoglycan in nervous tissue. I. Adult brain, retina, and peripheral nerve. J. Cell Biol. 99(3):1117-1129.

Ashwell G, Harford J. 1982. Carbohydrate-specific receptors of the liver. Annual Reviews in Biochem. J 51(1):531-554.

Baik JY, Lee MS, An SR, Yoon SK, Joo EJ, Kim YH, Park HW, and Lee GM. Initial transcriptome and proteome analyses of low culture temperature-induced expression in CHO cells producing erythropoietin. Biotech. Bioeng. 93(2): 361-371.

Baker KN, Rendall MH, Hills AE, Hoare M, Freedman RB, James DC. 2001. Metabolic control of recombinant protein N-glycan processing in NS0 and CHO cells. Biotechnol. Bioeng. 73(3):188-202.

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

Bavarian F, Fan LS, J.J.Chalmers. 1991. Microscopic visualization of insect cell-bubble interactions. I. Rising bubbles, air-medium interface, and the foam layer. Biotechnol. Progr. 7:140-50.

Bentley PK, Gates R, Lowe K. 1989. In vitro cellular responses to a non-ionic surfactant, Pluronic F68. Biotechnol. Lett 11:111-114.

Bereiter-Hahn J, Luck M, Miebach T. 1990. Spreading of trypsinized cells: Cytoskeletal dynamics and energy requirements. Journal of Cell Science 96:171-188.

Berry JM, Barnabe N, Coombs KM, Butler M. 1999. Production of Reovirus type-1 and type-3 from Vero cells grown on solid and macroporous microcarriers. Biotechnol. Bioeng. 62(1):12-19.

Bigge JC, Patel TP, Bruce JA, Goulding PN, Charles SM, Parekh RB. 1995. Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Analytical Biochem. J 230(2):229-238.

Biran R, Noble MD, Tresco PA. 1999. Characterization of cortical astrocytes on materials of differing surface chemistry. J. Biomed. Mater. Res.46:150-159.

Bleckwenn NA, Golding H, Bentley WE, Shiloach J. 2005. Production of recombinant proteins by vaccinia virus in a microcarrier based mammalian cell perfusion bioreactor. Biotechnol. Bioeng. 90(6):663-674.

Borys MC, Linzer DIH, Papoutsakis ET. 1993. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. Bio/Technology 11(6):720-724.

Brooks SA. 2004. Appropriate glycosylation of recombinant proteins for human use: Implications of choice of expression system. Mol. Biotechnol. 28(3):241.

Browne S, Al-Rubeai M. 2007. Selection methods for high-producing mammalian cell lines. TIBS. 25(9): 425-432.

Burridge K, Chrzanowska-Wodnicka M. 1996. Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol. 12(1):463-519.

Butler M. 2004. Mammalian cell culture and technology. Butler M, editor. Oxford, England: Oxford University Press.

Butler M. 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl Microbiol Biotechnol 68(3):283-91.

Butler M. 2007. Cell line development and culture strategies: future prospects to improve yields. In: Butler M, editor. Cell Culture and Upstream Processing. New York, NY: Taylor & Francis Group.

Butler M, Huzel N, Barnabe N, Gray T, Bajno L. 1999. Linoleic acid improves the robustness of cells in agitated cultures. Cytotechnol. 30:27-36.

Calder PC, Yaqoob P, Harvey DJ, Watts A, Newsholme EA. 1994. Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. Biochem. J. 300(2):509-518.

Campbell CT, Sampathkumar SG, Yarema KJ. 2007. Metabolic oligosaccharide engineering: perspectives, applications, and future directions. Mol. Biosyst. 3(3):187-194.

Castro PML, Ison AP, Hayter PM, Bull AT. 1995. CHO cell growth and recombinant interferon-γ production: Effects of BSA, Pluronic and lipids. Cytotechnol. 19(1):27-36.

Chalmers JJ, Bavarian F. 1991. Microscopic visualization of insect cell-bubble interactions. II. The bubble film and bubble rupture. Biotechnol. Progr. 7:151-158.

Chandaroy P, Sen A, Alexandridis P, Hui SW. 2002. Utilizing temperature-sensitive association of Pluronic F-127 with lipid bilayers to control liposome-cell adhesion. Biochim. Biophys. Acta 1559:32-42.

Chattopadhyay D, Rathman JF, Chalmers JJ. 1995. The protective effect of specific medium additives with respect to bubble rupture. Biotechnol. Bioeng. 45:473-480.

Chen Z, Benchuan W, Hong L, Shichong L, Peitang H. 2000. Study of rt-PA production by recombinant CHO cells immobilized with a porous microcarrier. Chin. J. Biotechnol. 15(4):239-244.

Chen Z, Lütkemeyer D, Iding K, Lehmann J. 2001. High-density culture of recombinant Chinese hamster ovary cells producing prothrombin in protein-free medium. Biotechnol. Lett 23(10):767-770.

Chen Z, Wu B, Liu H, Liu X, Huang P. 2004. Temperature shift as a process optimization step for the production of pro-urokinase by a recombinant Chinese hamster ovary cell line in high-density perfusion culture. J. Biosci. Bioeng. 97(4):239-243.

Cherry RS, Hulle CT. 1992. Cell death in the thin films of bursting bubbles. Biotechnol. Progr. 8:11-18.

Chevalot I, Visvikis A, Nabet P, Engasser J-M, Marc A. 1994. Production of a membrane-bound protein, the human gamma-glutamyl transferase, by CHO cells cultivated on microcarriers, in aggregates and suspension. Cytotechnol. 16:121-129.

Chisti Y. 1999. Shear Sensitivity. In: Flickinger MCaD, S.W., editor. Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation. New York: Wiley. p 2379-2406.

Choi BK, Bobrowicz P, Davidson RC, Hamilton SR, Kung DH, Li H, Miele RG, Nett JH, Wildt S, Gerngross TU. 2003. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. Proceedings of the National Academy of Sciences 100(9):5022.

Chothia C, Jones EY. 1997. The molecular structure of cell adhesion molecules. Annual Review of Biochem. J 66(1):823-862.

Chotigeat, W., Watanapokasin, Y., Mahler, S., Gray, P.P., 1994. Role of environmental conditions on the expression levels, glycoform pattern and levels of sialyltransferase for hFSH produced by recombinant CHO cells. Cytotechnology 15, 217–221.

Chuppa S, Tsai YS, Yoon S, Shackleford S, Rozales C, Bhat R, Tsay G, Matanguihan C, Konstantinov K, Naveh D. 1997. Fermentor temperature as a tool for control of high-density perfusion cultures of mammalian cells. Biotechnol. Bioeng. 55(2):328-338.

Clark KJR, Chaplin FWR, Harcum SW. 2004. Temperature effects on product-quality-related enzymes in batch CHO cell cultures producing recombinant tPA. Biotechnol. Progr. 20(6):1888-1892.

Cong C, Chang Y, Deng J, Xiao C, Zhiguo S. 2001. A novel scale-up method for mammalian cell culture in packed-bed bioreactor. Biotechnol. Lett. 23:881-885.

Conradt HS, Egge H, Peter-Katalinic J, Reiser W, Siklosi T, Schaper K. 1987. Structure of the carbohydrate moiety of human interferon-beta secreted by a recombinant Chinese hamster ovary cell line. J. Biol. Chem. 262(30):14600-14605.

Cromwell MEM, Hilario E, Jacobson F. 2006. Protein aggregation and bioprocessing. The AAPS Journal. 8(3):572-579.

Cruz HJ, Dias EM, Moreira JL, and Carrondo MJT. 1997. Cell-dislodging methods under serum-free conditions. Appl. Microbiol. Biotechnol. 47(5):482-488.

Cumming DA. 1991. Glycosylation of recombinant protein therapeutics: control and functional implications. Glycobiology 1(2):115-130.

Curling EM, Hayter PM, Baines AJ, Bull AT, Gull K, Strange PG, Jenkins N. 1990. Recombinant human interferon-gamma. Differences in glycosylation and proteolytic processing lead to heterogeneity in batch culture. Biochemical Journal 272(2):333.

de Boer L, Gray PP and Sunstrom N-A. 2004. Enhanced productivity of G1 phase Chinese hamster ovary cells using the GADD153 promoter. Biotechnol. Lett. 26(1): 61-65.

Detrait E, Lhoest JB, Bertrand P, Aguilar PBd. 1999. Fibronectin-pluronic co-adsorption on a polystyrene surface with increasing hydrophobicity: relationship to cell adhesion. Journal of Biomedical Materials Reseach 45:404-413.

Dewez J-L, Lhoest J-B, Detrait E, Berger V, Dupont-Gillain CC, Vincent L-M, Schneider Y-J, Bertrand P, Rouxhet PG. 1998. Adhesion of mammalian cells to polymer surfaces from physical chemistry of surfaces to selective adhesion on defined patterns. Biomaterials 19:1441-1445.

Dewez J, Doren A, Schnieder Y, Rouxhet P. 1999. Competitve adsorption of proteins: Key of the relationship between substratum surface properties and adhesion of epithelial cells. Biomaterials 20:547-559.

Dewez J, Schnieder Y, Rouxhet P. 1996. Coupled influence of substratum hydrophobicity and surfactant on epithelial cell adhesion. J. Biomed. Mater. Res.30:373-83.

Dey D, Boulton-Stone JM, Emery AN, Blake JR. 1997. Experimental comparisons with numerical model of surfactant effects on the burst of a single bubble. Chemical Engineering Science 52(16):2769-2783.

deZengotita VM, Abston LR, Schmelzer AE, Shaw S, Miller WM. 2002. Selected amino acids protect hybridoma and CHO cells from elevated carbon dioxide and osmolality. Biotechnol. Bioeng. 78(7):741-52.

Diaz S, Higa HH, Hayes BK, Varki A. 1989. O-acetylation and de-O-acetylation of sialic acids. 7-and 9-o-acetylation of alpha 2, 6-linked sialic acids on endogenous N-linked glycans in rat liver Golgi vesicles. J. Biol. Chem. 264(32):19416-19426.

Duerrschmid M, Landauer K, Simic G, Blueml G, Doblhoff-Dier O. 2003. Scalable inoculation strategies for microcarrier-based animal cell bioprocesses. Biotechnol. Bioeng. 83(6):681-686.

Dutton RL, Scharer J, Moo-Young M. 2006. Cell cycle phase dependent productivity of a recombinant Chinese hamster ovary cell line. Cytotechnol. 52(1):55-69.

Einarsson M, Brandt J, Kaplan, L. 1985. Large-scale purification of human tissue-type plasminogen activator using monoclonal antibodies. Biochim Biophys Acta 830:1–10.

Fann CH, Guirgis F, Chen G, Lao MS, Piret JM. 2000. Limitations to the amplification and stability of human tissue-type plasminogen activator expression by Chinese hamster ovary cells. Biotechnol. Bioeng. 69(2):204-212.

Fogolin MB, Wagner R, Etcheverrigaray M, Kratje R. 2004a. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. J. Biotechnol. 109(1-2):179-191.

Fox SR, Patel UA, Yap MGS, Wang DIC. 2004. Maximizing Interferon– ɣ production by Chinese hamster ovary cells through temperature shift optimization. Biotechnol. Bioeng. 85(2).

Fox SR, Tan HK, Tan MC, Wong S, Yap MGS, Wang DIC. 2005. A detailed understanding of the enhanced hypothermic productivity of interferon-ɣ by Chinese-hamster ovary cells. Biotechnol. Appl. Biochem. 41:255-264.

Frazzati-Gallina NM, Paoli RL, Mourao-Fuches RM, Jorge SAC, Pereira CA. 2001. Higher production of rabies virus in serum-free medium cell cultures on microcarriers. J. Biotechnol. 92(1):67-72.

Furukawa K, Ohsuye K. 1998. Effect of culture temperature on a recombinant CHO cell line producing a C-terminal a-amidating enzyme. Cytotechnol. 26(2):153-164.

Gawlitzek M, Valley U, Nimtz M, Wagner R, Conradt HS. 1995. Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21 cells due to different culture conditions. J. Biotechnol. 42(2):117-131.

GE-Healthcare. 2002. Cytopore - macroporous microcarriers. Sweden: Wikstroms.

GE-Healthcare. 2005. Microcarrier cell culture: principles and methods: Elanders Tofters. p. 61-72

Ghebeh H. 2002. The effect of the non-ionic surfactant Pluronic F68 on mammalian cells in culture [Ph.D.]. Winnipeg: University of Manitoba.

Ghebeh H, Gillis J, Butler M. 2002. Measurement of hydrophobic interactions of mammalian cells grown in culture. J. Biotechnol. 95(39-48).

Gigout A, Buschmann MD, Jolicoeur M. 2008. The fate of Pluronic F-68 in chondrocytes and CHO cells. Biotechnol. Bioeng. .

Ginsberg MH, Partridge A, Shattil SJ. 2005. Integrin regulation. Curr. Opin. Cell Biol. 17(5):509-516.

Goldblum S, Bae Y, Hink F, Chalmers J. 1990. Protective effect of methylcellulose and other polymers on insect cells subjected to laminar shear stress. Biotechnol. Progr. 6(383-390).

Goldman MH, James DC, Rendall M, Ison AP, Hoare M, Bull AT. 1998. Monitoring recombinant human interferon-gamma N-glycosylation during perfused fluidized-bed and stirred-tank batch culture of CHO cells. Biotechnol. Bioeng. 60(5):596-607.

Goldwasser E, Kung CKH, Eliason J. 1974. On the Mechanism of Erythropoietin-induced differentiation XIII. The role of sialic acid in Erythropoietin action. J. Biol. Chem. 249(13):4202-4206.

Gomord V, Chamberlain P, Jefferis R, Faye L. 2005. Biopharmaceutical production in plants: problems, solutions and opportunities. Trends Biotechnol. 23(11):559-565.

Gramer MJ, Goochee CF. 1993. Glycosidase activities in Chinese hamster ovary cell lysate and cell culture supernatant. Biotechnol. Progr. 9(4):366-373.

Grinnell F. 1978. Cellular adhesiveness and extracellular substrata. Int. Rev. Cytol. 53:65-144.

Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, Nett JH, Rausch S, Stadheim TA, Wischnewski H. 2003. Production of complex human glycoproteins in yeast. Science. 301(5637):1244.

Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK. 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. Am. Assoc. Adv. Sci. Pub. p 1441-1443.

Handa-Corrigan A, Emery AN, Spier RE. 1989. Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubble. Enzyme Microb. Technol. 11:230-235.

Hayter PM, Curling EMA, Baines AJ, Jenkins N, Salmon I, Strange PG, Tong JM, Bull AT. 1992. Glucose-limited chemostat culture of chinese hamster ovary cells producing recombinant human interferon-gamma. Biotechnol. Bioeng. 39(3):327-335.

Hevey M, Negley D, Geisbert J, Jahrling P, Schmaljohn A. 1997. Antigenicity and vaccine potential of Marburg Virus glycoprotein expressed by Baculovirus recombinants. Virology 239(1):206-216.

Hokke CH, Bergwerff AA, Vandedem GWK, Kamerling JP, and Vliegenthart JFG. 1995. Structural-analysis of the sialylated N-linked and O-linked carbohydrate chains of recombinant-human-erythropoeitin expressed in Chinese hamster ovary cells-sialylation patterns and branch location of dimeric N-acteyllactosamine units. Eur. J. Biochem. 228: 981-1008.

Hu X, Xiao C, Huang Z, Guo Z, Zhang Z, Li Z. 2000. Pilot production of u-PA with porous microcarrier cell culture. Cytotechnol. 33(1):13-19.

Hughes RC, Pena SD, Clark J, Dourmashkin RR. 1979. Molecular requirements for adhesion and spreading of hamster fibroblasts. Exp. Cell. Res. 121(2):307-14.

Ikonomou L, Drugmand J-C, Bastin G, Schneider Y-J, Agathos SN. 2002. Microcarrier culture of Lepidopteran cell lines: Implications for growth and recombinant protein production. Biotechnol. Progr. 18:1345-55.

Imai N, Higuchi M, Kawamura A, Tomonoh K, Oh-Eda M, Fujiwara M, Shimonaka Y, Ochi N. 1990. Physicochemical and biological characterization of asialoerythropoietin. Suppressive effects of sialic acid in the expression of biological activity of human erythropoietin in vitro. Eur. J. Biochem. 194(2):457-462.

Jayme DW. 1991. Nutrient optimization for high density biological production applications. Cytotechnol. 5(1):15-30.

Jenkins N, Parekh RB, James DC. 1996. Getting the glycosylation right: Implications for the biotechnology industry. Nature Biotechnol. 14(8):975-981.

Joosten CE, Shuler ML. 2003. Effect of culture conditions on the degree of sialylation of a recombinant glycoprotein expressed in insect cells. Biotechnol. Prog 19(3):739–749.

Jordan M, Sucker H, Einsele A, Widmer F, Eppenberger HM. 1994. Interactions between animal cells and gas bubbles: The influence of serum and Pluronic F68 on the physical properties of the bubble surface. Biotechnol. Bioeng. 43:446-454.

Jorjani P, Ozturk SS. 1999. Effects of cell density and temperature on oxygen consumption rate for different mammalian cell lines. Biotechnol. Bioeng. 64(3):349-356.

Kagawa Y, Takasaki S, Utsumi J, Hosoi K, Shimizu H, Kochibe N, Kobata A. 1988. Comparative study of the asparagine-linked sugar chains of natural human interferon-beta 1 and recombinant human interferon-beta 1 produced by three different mammalian cells. J. Biol. Chem. 263(33):17508-17515.

Kallel H, Rourou S, Majoul S, Loukil H. 2003. A novel process for the production of a veterinary rabies vaccine in BHK-21 cells grown on microcarriers in a 20-l bioreactor. Appl. Microbiol. Biotechnol. 61(5):441-446.

Kaparakis G, Alderson PG. 2003. Enhancement of in vitro cell proliferation of pepper (*Capsicum annuum L.*) by Pluronic F-68, haemoglobin and arabinogalactan proteins. J Hortic. Sci. Biotechnol. 78(5):647-649.

Karpusas M, Nolte M, Benton CB, Meier W, Lipscomb WN, Goelz S. 1997. The crystal structure of human interferon beta at 2.2-A resolution. Proc. Nat. Acad. Sci. 94(22):11813.

Karpusas M, Whitty A, Runkel L, Hochman P. 1998. The structure of human interferon-ß: implications for activity. Cell. Mol. Life. Sci. 54(11):1203-1216.

Katakam M, Ravis WR, Golden DL, Banga AK. 1997. Controlled release of human growth hormone following subcutaneous administration in dogs. Int. J. Pharm. 152(1):53-58.

Keane JT, Ryan D, Gray PP. 2003. Effect of shear stress on expression of a recombinant protein by Chinese hamster ovary cells. Biotechnol. Bioeng. 81(2):211-220.

Keen MJ, Rapson NT. 1995. Development of a serum-free culture medium for the large scale production of recombinant protein from a Chinese hamster ovary cell line. Cytotechnol. 17:153-163.

Keenan J, Pearson D, Clynes M. 2006. The role of recombinant proteins in the development of serum-free media. Cytotechnol. 50(1):49-56.

Khmelnitsky YL. 2004. Current strategies for in vitro protein glycosylation. J. Mol. Catal. B: Enzym. 31(4-6):73-81.

Kilburn D, Webb F. 2000. The cultivation of animal cells at controlled dissolved oxygen partial pressure. Reprinted from Biotechnol. Bioeng. Vol. X, Issue 6, Pages 801-814 (1968). Biotechnol. Bioeng. 67(6):657-670.

Kim D, Lee YJ. 1993. Effect of glycerol on protein aggregation: quantification of thermal aggregation of proteins from CHO cells and analysis of aggregated proteins. J. Therm. Biol. 18(1):41-48.

Kim SJ, Kim NS, Ryu CJ, Hong HJ, Lee GM. 1998. Characterization of chimeric antibody producing CHO cells in the course of dihydrofolate reductase-mediated gene amplification and their stability in the absence of selective pressure. Biotech. Bioeng. 58(1): 73-84.

Kimura R, Miller WM. 1997. Glycosylation of CHO-derived recombinant tPA produced under elevated pCO_2. Biotechnol. Progr. 13(3):311-7.

Kleinman HK, Klebe RJ, Martin GR. 1981. Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol. 88:473-485.

Kong D, Cardak S, Chen M, Gentz R, Zhang J. 1999a. High cell density and productivity culture of Chinese hamster ovary cells in a fluidized bed bioreactor. Cytotechnol. 29(3):215-220.

Kong D, Chen M, Gentz R, Zhang J. 1999b. Cell growth and protein formation on various microcarriers. Cytotechnol. 29(2):149-156.

Kontoravdi C, Asprey SP, Pistikopoulos EN, Mantalaris A. 2007. Development of a dynamic model of monoclonal antibody production and glycosylation for product quality monitoring. Comput. Chem. Eng. 31(5-6):392-400.

Kornfeld S, Kornfeld R, Neufeld EF, O'Brien PJ. 1964. The feedback control of sugar nucleotide biosynthesis in liver. Proc. Nat. Acad. Sci. U.S.A. 52(2):371-379.

Kunas KT, Papoutsakis ET. 1990. Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment. Biotechnol. Bioeng. 36:476-483.

Kunkel JP, Jan DCH, Jamieson JC, Butler M. 1998. Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody. J. Biotechnol. 62(1):55-71.

Kyung YS, Peshwa MV, Gryte DM, Hu WS. 1994. High density culture of mammalian cells with dynamic perfusion based on on-line oxygen uptake rate measurements. Cytotechnol. 14(3):183-190.

Laemmli U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277:680-685.

Lakhotia S, Bauer KD, Papoutsakis ET. 1993. Fluid-mechanical forces in agitated bioreactors reduce the CD13 and CD33 surface protein content of HL60 cells. Biotechnol. Bioeng. 41:868-877.

Lee SY, Kim DI. 2002. Stimulation of murine granulocyte macrophage-colony stimulating factor production by Pluronic F-68 and polyethylene glycol in transgenic *Nicotiana tabacum* cell culture. Biotechnol. Lett 24(21):1779-1783.

LeFloch F, Tessier B, Chenuet S, Guillaume JM, Cans P, Goergen JL, Marc A. 2006. Related effects of cell adaptation to serum-free conditions on murine EPO production and glycosylation by CHO cells. Cytotechnol. 52(1):39-53.

Legate KR, Montanez E, Kudlacek O, Fassler R. 2006. ILK, PINCH and parvin: the tIPP of integrin signalling. Nat. Rev. Mol. Cell Biol 7:20–31.

Levy ML, Luu T, Meltzer HS, Bennett R, Bruce DA. 2004. Bacterial adhesion to surfactant-modified silicone surfaces. Neurosurgery 54:488-491.

Lim HS, Han BK, Kim JH, Peshwa MV, Hu WS. 1992. Spatial distribution of mammalian cells grown on macroporous microcarriers with improved attachment kinetics. Biotechnol. Progr. 8(6):486-493.

Lin AA, Kimura R, Miller WM. 1993. Production of tPA in recombinant CHO cells under oxygen-limited conditions. Biotechnol. Bioeng. 42(3):339-350.

Liu CC, Wu SC. 2004. Mosquito and mammalian cells grown on microcarriers for four-serotype dengue virus production: Variations in virus titer, plaque morphology, and replication rate. Biotechnol. Bioeng. 85(5):482-488.

Liu VA, Jastromb WE, Bhatia SN. 2002. Engineering protein and cell adhesivity using PEO-terminated triblock polymers. J. Biomed. Mater. Res. 60:126-134.

Ludwig A, Kretzmer G, Schugerl K. 1992. Determination of a "critical shear stress level" applied to adherent mammalian cells. Enzyme Microb. Technol. 14:209-213.

Ma JKC, Hein MB. 1995. Immunotherapeutic potential of antibodies produced in plants. Trends Biotechnol. 13(12):522-527.

Ma N, Chalmers JJ, Aunins JG, Zhou W, Xie L. 2004. Quantitative studies of cell-bubble interactions and cell damage at different pluronic F-68 and cell concentrations. Biotechnol. Progr. 20(4):1183-91.

Mallik A, Pinkus GS, Sheffer. S. 2002. Biopharma's capacity crunch. The McKinsey Quarterly. 2002 Special Edition: Risk and Resilience: McKinsey & Company. p 9-11.

Marquis CP, Low KS, Barford JP, Harbour C. 1989. Agitation and aeration effects in suspension mammalian cell cultures. Cytotechnol. 2:163-170.

Masse T, Kelly PT. 1997. Overexpression of Ca2+/calmodulin-dependent protein kinase II in PC12 cells alters cell growth, morphology, and nerve growth factor-induced differentiation. J. Neurosci. 17(3):924.

Meier SJ, Hutton A, Wang DIC. 1999. Cell-bubble attachment and sparging-related death. Biotechnol. Bioeng. 62(4):470-477.

Meng F, Park Y, Zhou H. 2001. Role of proline, glycerol, and heparin as protein folding aids during refolding of rabbit muscle creatine kinase. Int. J. Biochem. Cell Biol. 33(7):701-9.

Michaels JD, Nowak JE, Mallik AK, Koczo K, Wasan DT, Papoutsakis ET. 1995. Analysis of cell-to-bubble attachment in sparged bioreactors in the presence of cell-protecting additives. Biotechnol. Bioeng. 47:407-419.

Michaels JD, Petersen JF, McIntire LV, Papoutsakis ET. 1991. Protection mechanisms of freely suspended animal cells (CRL 8018) from fluid-mechanical injury. viscometric and bioreactor studies using serum, Pluronic F68 and polyethylene glycol. Biotechnol. Bioeng. 38:169-180.

Mignot G, Faure T, Ganne V, Arbeille B, Pavirani A, Romet-Lemonne JL. 1990. Production of recombinant Von Willebrand factor by CHO cells cultured in macroporous microcarriers. Cytotechnol. 4(2):163-171.

Mizrahi A. 1975. Pluronic polyols in human lymphocyte cell line cultures. J. Clin. Microbiol. 2:11-13.

Moore A, Mercer J, Dutina G, Donahue CJ, Bauer KD, Mather JP, Etcheverry T and Ryll T. Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultures.Cytotech 1997, 23:47-54.

Muething J, Kemminer SE, Conradt HS, Šagi D, Nimtz M, Kaerst U, Peter-Katalinic J. 2003. Effects of buffering conditions and culture pH on production rates and glycosylation of clinical phase I anti-melanoma mouse IgG 3 monoclonal antibody R 24. Biotechnol. Bioeng. 83(3):321-334.

Murakami H. 1990. What should be focused in the study of cell culture technology for production of bioactive proteins. Cytotechnol. 3(1):3-7.

Murhammer DW, Goochee CF. 1990. Sparged animal cell bioreactors: Mechanism of cell damage and Pluronic F-68 protection. Biotechnol. Progr. 6:391-39.

Nabi I, Dennis J. 1998. The extent of polylactosamine glycosylation of MDCK LAMP-2 is determined by its Golgi residence time. Glycobiology 8:947-53.

Nam JH, Ermonval M, Sharfstein ST. 2007a. Cell attachment to microcarriers affects growth, metabolic activity, and culture productivity in bioreactor culture. Biotechnol. Progr. 23(3):652-660.

Nam JH, Zhang F, Ermonval M, Linhardt RJ, Sharfstein S. 2007b. Effects of microcarrier culture and temperature shift on productivity and glycosylation of recombinant proteins produced in Chinese hamster ovary cells. The 2007 Annual Meeting AIChE.

Narhi LO, Arakawa T, Aoki KH, Elmore R, Rohde MF, Boone T, Strickland TW. 1991. The effect of carbohydrate on the structure and stability of erythropoietin. J. Biol. Chem. 266(34):23022-23026.

Ng YC, Berry JM, Butler M. 1996. Optimization of physical parameters for cell attachment and growth on macroporous microcarriers. Biotechnol. Bioeng. 50(6):627-635.

Nikolai TJ, Hu WS. 1992. Cultivation of mammalian cells on macroporous microcarriers. Enzyme Microb. Technol. 14(3):203-8.

Nishiyama H, Higashitsuji H, Yokoi H, Itoh K, Danno S, Matsuda T and Fujita J., Cloning and characterization of human CIRP (cold-inducible RNA-binding protein) cDNA and chromosomal assignment of the gene., Gene. 1997, 204(1-2):115-20.

Noguchi A, Mukuria CJ, Wang WC, and Raff HV. 1995. Immunogenicity of N-glycolylneuraminic acid -containing carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. J. Biochem. 117: 59-62.

Palomares LA, Gonzalez M, Ramirez OT. 2000. Evidence of Pluronic F-68 direct interaction with insect cells: impact on shear protection, recombinant protein, and baculovirus production. Enzyme Microb. Technol. 26:324-331.

Palsson B and Bhatia SN. 2003. Tissue Engineering. Palsson B, Hubbell JA, Plonsey R, Bronzino JD, editor. Boca Raton, USA: CRC Press.

Pfeiffer G, Strube KH, Schmidt M, Geyer R. 1994. Glycosylation of two recombinant human uterine tissue plasminogen activator variants carrying an additional N-glycosylation site in the epidermal-growth-factor-like domain. Eur. J. Biochem. 219(1-2):331-348.

Puck TT, Cieciura SJ, Robinson A. 1958. Genetics of Somatic Mammalian cells III. Long-term Cultivation of euploid cells from human and animal subjects. J. Exp. Med. 108(6):945-956.

Qiu J, Swartz JR, Georgiou G. 1998. Expression of active human tissue-type plasminogen activator in *Escherichia coli*. Appl. Environ. Microbiol. 64(12):4891-4896.

Raju TS, Briggs JB, Borge SM, Jones AJS. 2000. Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. Glycobiology 10(5):477-486.

Ramirez OT, Mutharasan R. 1990. The role of plasma membrane fluidity on the shear sensitivity of hybridoma grown under hydrodynamic stress. Biotechnol. Bioeng. 36:911-920.

Reingold SC. 1996. The future with interferons. MS Management 3(2):1-7.

Restelli V, Wang MD, Huzel N, Ethier M, Perreault H, Butler M. 2006. The effect of dissolved oxygen on the production and the glycosylation profile of recombinant human erythropoietin produced from CHO cells. Biotechnol. Bioeng. 94(3):481-494.

Ricci MS, Brems DN. 2004. Common structural stability properties of 4-helical bundle cytokines: possible physiological and pharmaceutical consequences. Curr. Pharm. Des. 10(31):3901-3912.

Rodriguez J. 2007. The development of a cell culture process for the production of recombinant human beta interferon from a transfected Chinese hamster ovary cell line. Ph.D. Thesis. Winnipeg: University of Manitoba. 227

Rodriguez J, Spearman M, Huzel N, Butler M. 2005. Enhanced production of monomeric interferon-beta by CHO cells through the control of culture conditions. Biotechnol Prog 21(1):22-30.

Rodriguez J, Tharmalingam T, Spearman M, Sunley K, Lodewycks C, Huzel N, and Butler M. 2008. The optimization of a perfusion culture for the production of recombinant interferon-beta *(In progress)*.

Ruffieux PA, von Stockar U, Marison IW. 1998. Measurement of volumetric (OUR) and determination of specific (qO_2) oxygen uptake rates in animal cell cultures. J. Biotechnol. 63(2):85-95.

Runkel L, Meier W, Pepinsky RB, Karpusas M, Whitty A, Kimball K, Brickelmaier M, Muldowney C, Jones W, Goelz SE. 1998. Structural and functional differences between glycosylated and non-glycosylated forms of human Interferon-ß (IFN-ß). Pharm. Res. 15(4):641-649.

Sakai K, Hayashi C, Yamaji H, Fukuda H. 2001. Use of nonionic surfactants for effective supply of phosphatidic acid in serum-free culture of Chinese hamster ovary cells. J. Biosci. Bioeng. 92(3):256-261.

Sakai K, Matsunaga T, Hayashi C, Yamaji H, Fukuda H. 2002. Effects of phosphatidic acid on recombinant protein production by Chinese hamster ovary cells in serum-free culture. Biochem. Eng. J. 10(2):85-92.

Schauer R. 1988. Sialic acids as antigenic determinants of complex carbohydrates. Adv. Exp. Med. Biol. 228:47-72.

Schneeberger EE, Lynch RD. 2004. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 286(6):1213-1228.

Schroder M, Matischak K, Friedl P. 2004. Serum- and protein-free media formulations for the Chinese hamster ovary cell line DUKXB11. J. Biotechnol. 108(3):279-92.

Schweikart F, Jones R, Jaton JC, Hughes GJ. 1999. Rapid structural characterisation of a murine monoclonal IgA a chain: heterogeneity in the oligosaccharide structures at a

specific site in samples produced in different bioreactor systems. J. Biotechnol. 69(2-3):191-201.

Senger RS, Karim MN. 2003. Effect of shear stress on intrinsic CHO culture state and glycosylation of recombinant tissue-type plasminogen activator protein. Biotechnol. Prog 19(4):1199–1209.

Serrato JA, Palomares LA, Meneses-Acosta A, Ramirez OT. 2004. Heterogeneous conditions in dissolved oxygen affect N-glycosylation but not productivity of a monoclonal antibody in hybridoma cultures. Biotechnol. Bioeng. 88(2):176-188.

Shi M, Xie Z, Yu M, Shen B, Guo N. 2005. Controlled growth of Chinese hamster ovary cells and high expression of antibody-IL-2 fusion proteins by temperature manipulation. Biotechnol. Lett 27(23):1879-1884.

Sinclair AM, Elliott S. 2005. Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. J. Pharm. Sci. 94(8):1626-35.

Sliwkowski MB, Gunson JV, Warner TG. 1992. Sialylation and mannose phosphorylation as a function of culture conditions for recombinant human deoxyribonuclease produced in CHO cells. J. Cell. Biochem. Suppl 16:150.

Smith CG, Greenfield, PF Randerson, DH. 1987. A technique for determining the shear sensitivity of mammalian cells in suspension culture. Biotechnol. Tech. 1(1):39-44.

Sowana DD, Williams DRG, O'Neill BK, Dunlop EH. 2002. Studies of the shear protective effects of Pluronic F-68 on wild carrot cell cultures. Biochem. Eng. J. 12(3):165-173.

Spearman M, Rodriguez J, Huzel N, Butler M. 2005. Production and glycosylation of recombinant beta-interferon in suspension and cytopore microcarrier cultures of CHO cells. Biotechnol. Progr. 21(1):31-9.

Stephens S, Emtage S, Vetterlein O, Chaplin L, Bebbington C, Nesbitt A, Sopwith M, Athwal D, Novak C, Bodmer M. 1995. Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses. Immunology 85(4):668–674.

Stephenson I, Wood JM, Nicholson KG, Zambon MC. 2003. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J. Med. Virol. 70(3):391-398.

Sun M-B, Jiang Y-J, Li W-D, Li P-Z, Li G-L, Jiang S-D, Liao G-Y. 2004. A novel process for production of hepatitus A virus in Vero cells grown on microcarriers in bioreactor. World. J. Gasteroenterol. 10(17):2571-2573.

Sun X, Linden, JC. 1999. Shear stress effects on plant cell suspension cultures in a rotating wall vessel bioreactor. J. Ind. Microbiol. Biotechnol. 22:44-47.

Tan HK, Lee MM, Yap MG, Wang DI. 2008. Overexpression of cold-inducible RNA-binding protein increases interferon-gamma production in Chinese-hamster ovary cells. Biotechnol. Appl. Biochem. 49(Pt 4):247-57.

Tan WS, Dai GC, Chen YL. 1994. Quantitative investigations of cell-bubble interactions using foam fractionation technique. Cytotechnol. 15:321-328.

Tharmalingam, T., Wuerz, T., Ghebeh, H., and Butler, M. 2008a. Pluronic Enhances the Robustness of Mammalian Cells and Reduces Surface Attachment. *Mol. Biotechnol.* 39(2), 167-77.

Tharmalingam, T., Sunley, K., Spearman, M., and Butler, M. 2008b. Enhanced Production of Recombinant Proteins by High Density Cell Entrapment in Microcarriers. (*In Progress)*

Tharmalingam, T., Sunley, K., and Butler, M. 2008c. High yields of monomeric recombinant beta-interferon from macroporous microcarrier cultures under hypothermic conditions. *Biotech. Prog.* 24(4):832-838.

Trinh K, Garcia-Briones M, Hink F, Chalmers JJ. 1994. Quantification of damage to suspended insect cells as a result of bubble rupture. Biotechnol. Bioeng. 43:37-45.

Trummer E, Fauland K, Seidinger S, Schriebl K, Lattenmayer C, Kunert R, Vorauer-Uhl K, Weik R, Borth N, Katinger H, and Müller D. 2006. Process parameter shifting: Part I. effect of DOT, pH and temperature on the performance of Epo-Fc expressing CHO cells cultivated in controlled batch bioreactors. Biotechnol. Bioeng. 94(6): 1033-1044.

Tsuiji H, Xu L, Schwartz K, Gumbiner BM. 2007. Cadherin conformations associated with dimerization and adhesion. J. Biol. Chem. 282(17):12871.

Utsumi J, Matsuo-Ogawa E, Nagahata T, Kasama K, Kagawa Y, Satoh Y. 1995. Carbohydrate-dependent biological activities of glycosylated human interferon-beta on human hepatoblastoma cells in vitro. Microbiol. Immunol.39(1):81-6.

Utsumi J, Yamazaki S, Kawaguchi K, Kimura S, Shimizu H. 1989. Stability of human interferon-beta 1: oligomeric human interferon-beta 1 is inactive but is reactivated by monomerization. Biochim. Biophys. Acta 998(2):167-72.

Van der Pol LaT, J. 1998. Shear sensitivity of animal cells from a culture-medium perspective. Trends Biotechnol. 16:323-328.

Virtanen I, Vartio T, Badley RA, Lehto VP. 1982. Fibronectin in adhesion, spreading and cytoskeletal organization of cultured fibroblasts. Nature 298(5875):660-663.

Wagner DD. 1986. Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. J. Cell Biol. 102(4):1320-1324.

Wang MD, Yang M, Huzel N, Butler M. 2002. Erythropoietin production from CHO cells grown by continuous culture in a fluidized-bed bioreactor. Biotechnol. Bioeng. 77(2):194-203.

Watson E, Shah B, Leiderman L, Hsu YR, Karkare S, Lu HS, Lin FK. 1994a. Comparison of N-linked oligosaccharides of recombinant human tissue kallikrein produced by Chinese hamster ovary cells on microcarrier beads and in serum-free suspension culture. Biotechnol Prog 10(1):39-44.

Weikert S, Papac D, Briggs J, Cowfer D, Tom S, Gawlitzek M, Lofgren J, Mehta S, Chisholm V, Modi N. 1999. Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. Nature Biotechnol. 17:1116-1121.

Weiss P, Ashwell G. 1989. The asialoglycoprotein receptor: properties and modulation by ligand. Prog. Clin. Biol. Res. 300:169-84.

Wen Z, Tan W. 1999. Quantitative measurements of cell-bubble attachment in bubble columns using foam flotation techniques. Biotechnol. Tech. 13:131-135.

Wiebe MG, Karandikar A, Robson GD, Trinci APJ, Candia JLF, Trappe S, Wallis G, Rinas U, Derkx PMF, Madrid SM. 2001. Production of tissue plasminogen activator (t-PA) in *Aspergillus niger*. Biotechnol. Bioeng. 76(2):164-174.

Wildt S, Gerngross TU. 2005. The humanization of N-glycosylation pathways in yeast. Nat. Rev. Microbiol. 3(2):119-128.

Wittwer AJ, Howard SC, Carr LS, Harakas NK, Feder J, Parekh RB, Rudd PM, Dwek RA, Rademacher TW. 1989. Effects of N-glycosylation on in vitro activity of Bowes melanoma and human colon fibroblast derived tissue plasminogen activator. Biochemistry. 28:7662–7669.

Wittwer AJ, Howard SC. 1990. Glycosylation at Asn-184 inhibits the conversion of single-chain to two-chain tissue-type plasminogen activator by plasmin. Biochem. J 29(17):4175-4180.

Wong DCF, Wong KTK, Goh LT, Heng CK, Yap MGS. 2005. Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol. Bioeng. 89:164-177.

Wu J. 1996. Insights into protective effects of medium additives on animal cells under fluid stresses: the hydrophobic interactions. Cytotechnol. 22:103-109.

Wu J, Ruan Q, Lam HYP. 1997. Effects of surface-active medium additives on insect cell surface hydrophobicity relating to cell protection against bubble damage. Enzyme Microb. Technol. 21:341-348.

Wu S-C, Hsieh W-C, Liau M-Y. 1998. Comparisons of microcarrier cell culture processes in one hundred mini-liter spinner flask and fifteen-liter bioreactor cultures. Bioprocess. Eng. 19:431-434.

Wu S-C, Huang GY-L, Liu J-H. 2002. Production of retrovirus and adenovirus vectors for gene therapy: A comparative study using microcarrier and stationary cell culture. Biotechnol. Prog. 18(3):617-622.

Wu SC, Liu CC, Lian WC. 2004. Optimization of microcarrier cell culture process for the inactivated enterovirus type 71 vaccine development. Vaccine 22(29):3858-3864.

Wurm FM. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22(11):1393-8.

Wyss DF, Wagner G. 1996. The structural role of sugars in glycoproteins. Curr. Opin. Biotechnol. 7(4):409-416.

Xiao C, Huang Z, Li W, Hu X, Qu W, Gao L, Liu G. 1998. High density and scale-up cultivation of recombinant CHO cell line and Hybridomas with porous microcarrier Cytopore. New develop. and New Applic. in Animal Cell Tech.:389-93.

Xiao C, Huang Z, Li W, Hu X, Qu W, Gao L, Liu G. 1999. High density and scale-up cultivation of recombinant CHO cell line and hybridomas with porous microcarrier Cytopore. Cytotechnol. 30(1):143-147.

Yamada K, Miyamoto S. 1995. Integrin transmembrane signaling and cytoskeletal control. Curr. Opin. Biotechnol. 7:681-89.

Yamaguchi M, Shirai Y, Inouye Y, Shoji M, Kamei M, Hashizume S, Shirahata S. 1997. Changes in monoclonal antibody productivity of recombinant BHK cells immobilized in collagen gel particles. Cytotechnol. 23(1):5-12.

Yang M, Butler M. 2000a. Effect of ammonia on the glycosylation of human recombinant erythropoietin in culture. Biotechnol. Prog 16(5):751-759.

Yang M, Butler M. 2000b. Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation. Biotechnol. Bioeng. 68(4):370.

Yang M, Butler M. 2002. Effects of ammonia and glucosamine on the heterogeneity of erythropoietin glycoforms. Biotechnol. Progr. 18(1):129-138.

Yokomizo AY, Antoniazzi MM, Galdino PL, Azambuja N, Jorge SAC, Pereira CA. 2004. Rabies virus production in high vero cell density cultures on macroporous microcarriers. Biotechnol. Bioeng. 85(5):506-515.

Yon JM. 2001. Protein folding: A perspective for biology, medicine and biotechnology. Braz J Med Biol Res 34(4):419-35.

Yoon SK, Hong JK, Choo SH, Song JY, Park HW, Lee GM. 2006. Adaptation of Chinese hamster ovary cells to low culture temperature: Cell growth and recombinant protein production. J. Biotechnol. 122:463-472.

Yoon SK, Hwang SO, Lee GM. 2004. Enhancing effect of low culture temperature on specific antibody productivity of recombinant Chinese hamster ovary cells: Clonal variation. Biotechnol. Progr. 20(6):1683-8.

Yoon SK, Kim SH, Lee GM. 2003. Effect of low culture temperature on specific productivity and transcription level of anti-4-1BB antibody in recombinant Chinese hamster ovary cells. Biotechnol. Progr. 19(4):1383-1386.

Yoshida H, Yoshizawa T, Shibasaki F, Shoji S, Kanazawa I. 2002. Chemical chaperones reduce aggregate formation and cell death caused by the truncated Machado-Joseph disease gene product with an expanded polyglutamine stretch. Neurobiol. Dis. 10(2):88-99.

Yun S-I, Yahya ARM, Cossar D, Anderson WA, Scharer JM, and Moo-Young M. 2004. Temperature downshift increases recombinant cytokine titer in Streptomyces lividans fermentation. Biotechnol. Lett. 23(3): 1903-1905.

Zhang Z, Al-Rubeai M, Thomas CR. 1992. Effect of Pluronic F-68 on the mechanical properties of mammalian cells. Enzyme Microb. Technol. 14:980-983.

Zhang Z, Chisti Y, and Moo-Young M. 1995. Effects of the hydrodynamic environment and shear protectants on survival of erythrocytes in suspension. J. Biotechnol. 43(1): 33-40.

Zhaolie C, Benchuan W, Hong L, Shichong L, Peitang H. 1999. Study on rt-PA production by recombinant CHO cells immobilized with a porous microcarrier. Chin. J. Biotechnol. 15(4):239-44.

Appendix A

Protein Detection

A.1 Introduction

The two recombinant proteins used for the duration of my research project were recombinant human IFN-beta and recombinant human tPA. Two CHO cell lines capable of producing these proteins were used as model systems for the study.

A.2. IFN-beta aggregation

The primary structure of IFN-beta reveals a high number of surface-exposed hydrophobic residues (such as Phe-70, Phe-154, Trp-79, and Trp-143) in the vicinity of the glycosylation site (Karpusas *et al.* 1997). In the absence of the glycan, the protein is more prone to aggregation, due to the possibility for the hydrophobic residues to interact with each other to stabilize the core of the molecule. The high intra molecular aggregation was found to decrease protein titres detected in the later stages of culture. The decrease in protein titres are usually consistent with increased turbidity (due to multimolecular aggregates) in the cell free supernatant.

Figure A.1 shows the typical cell growth and protein production from the IFN-beta producing CHO cells in a control suspension culture. From the initiation of the culture we can see an increase in protein in both the native protein and denatured protein yields until day 3. At days 4 and 5, there is an increase in both non-denatured and denatured titres. However at day 5, the native protein concentration reaches a maximum, and native

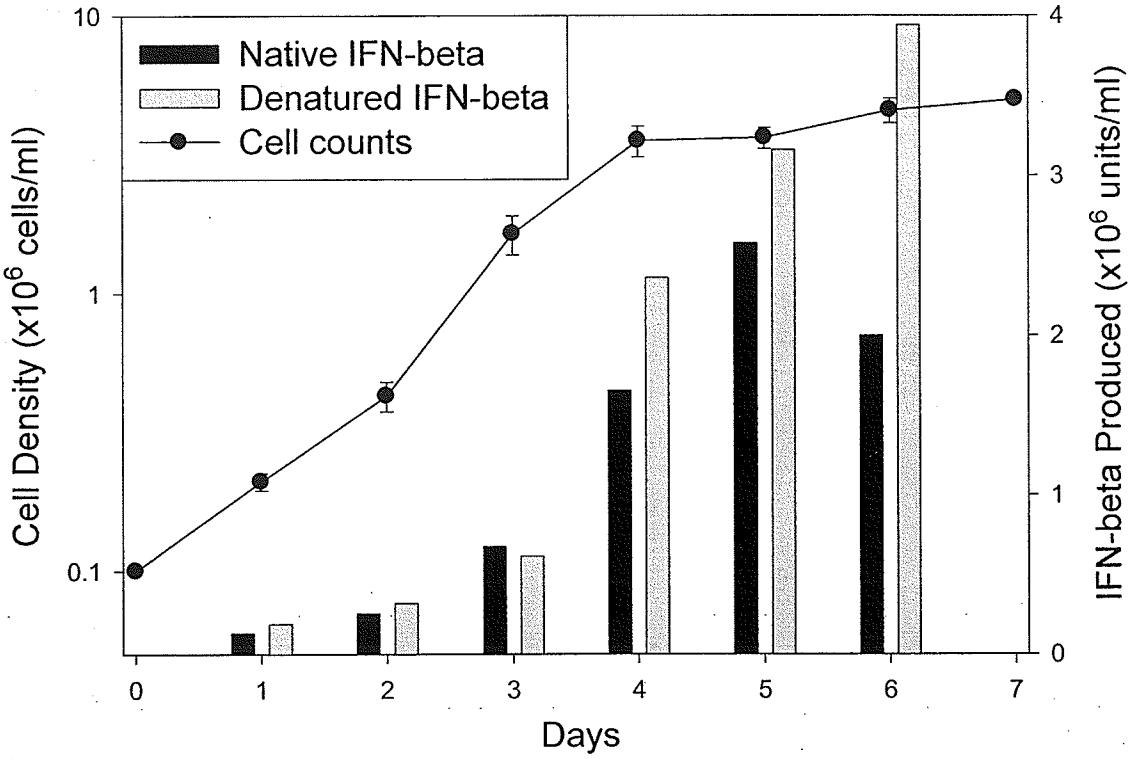


Figure A.1: The growth and productivity of CHO cells producing recombinant IFN-beta. The protein titres detected after day 5 decrease, as aggregation increases. The values represent means of duplicate samples ± SEM.

protein titres drop about 20 % at day 6. When the denatured protein is measured, the yield is found to increase further to just under $4x10^6$ units/mL from $3.2x10^6$ units/mL, the day before. The IFN-beta aggregation issue was the focus of a thesis project by Rodriguez (2007), and results presented in this appendix here are confirmatory results to show the aggregation problem is consistent.

The aggregation is reported to be due to multiple factors within a batch culture. Rodriguez (2007) reported that within a culture, temperature, protein concentration and residence time are factors that led to IFN-beta aggregation. Furthermore the use of low temperatures for the duration of a culture led to lower percentages of aggregation (Rodriguez *et al.* 2005). The temperature for storage of the IFN-beta samples as well as repeated freeze-thawing were important factors for the aggregation of samples, where protein aggregation was increased with higher storage temperatures (Figure A.2.) and repeated freeze-thawing (Rodriguez 2007).

A.3. tPA degradation

Figure A.3 shows the degradation of tPA. The tPA concentration measured by ELISA shows an increase in tPA to day 6 of the culture. However at day 7 we see a decrease of about 20 % in the protein titre compared to day 6. A western blot was done to confirm that the decrease in protein titre was not an artifact caused by aggregation. The western confirmed that the decrease was due to degradation of the protein. As the days progressed, the tPA band appeared to intensify, until day 5, after which the band was less intense. The band at 70 kDa appeared to decrease in intensity after day 5, and there was

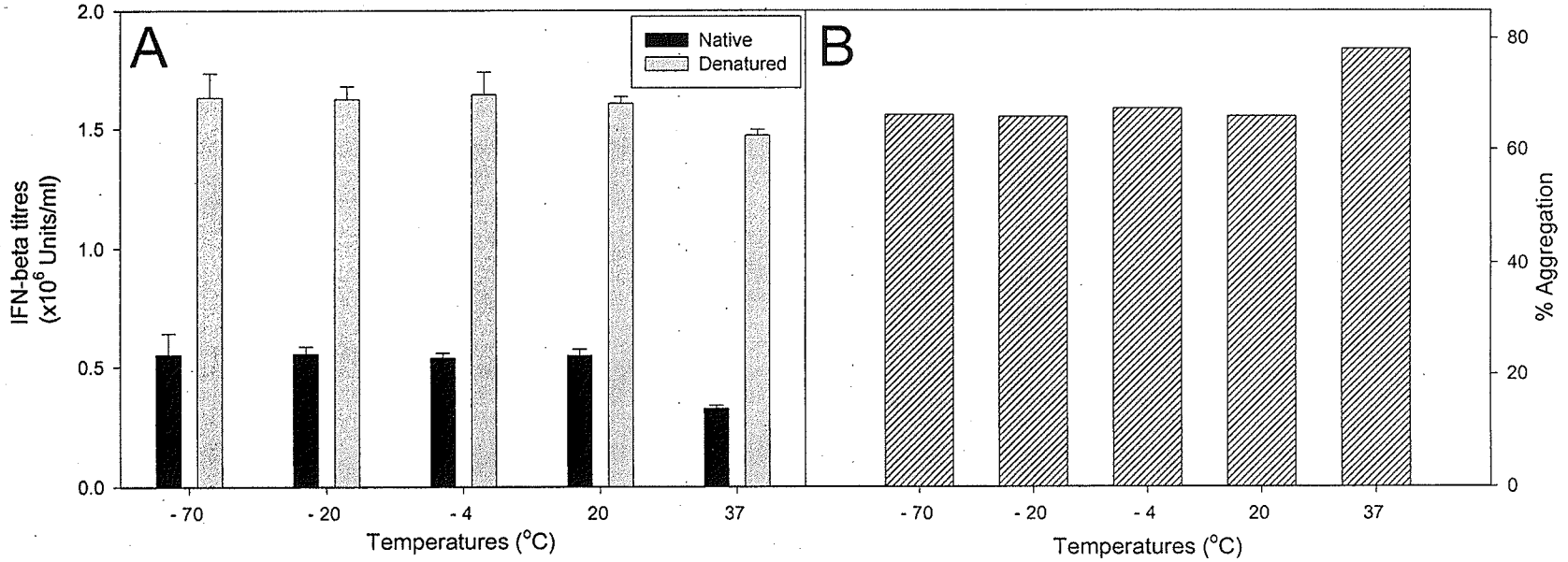


Figure A.2: Native and denatured IFN-beta titres at various storage temperatures (A). Samples were assayed after 48 hours at the storage temperature. The percent aggregation is represented in B.

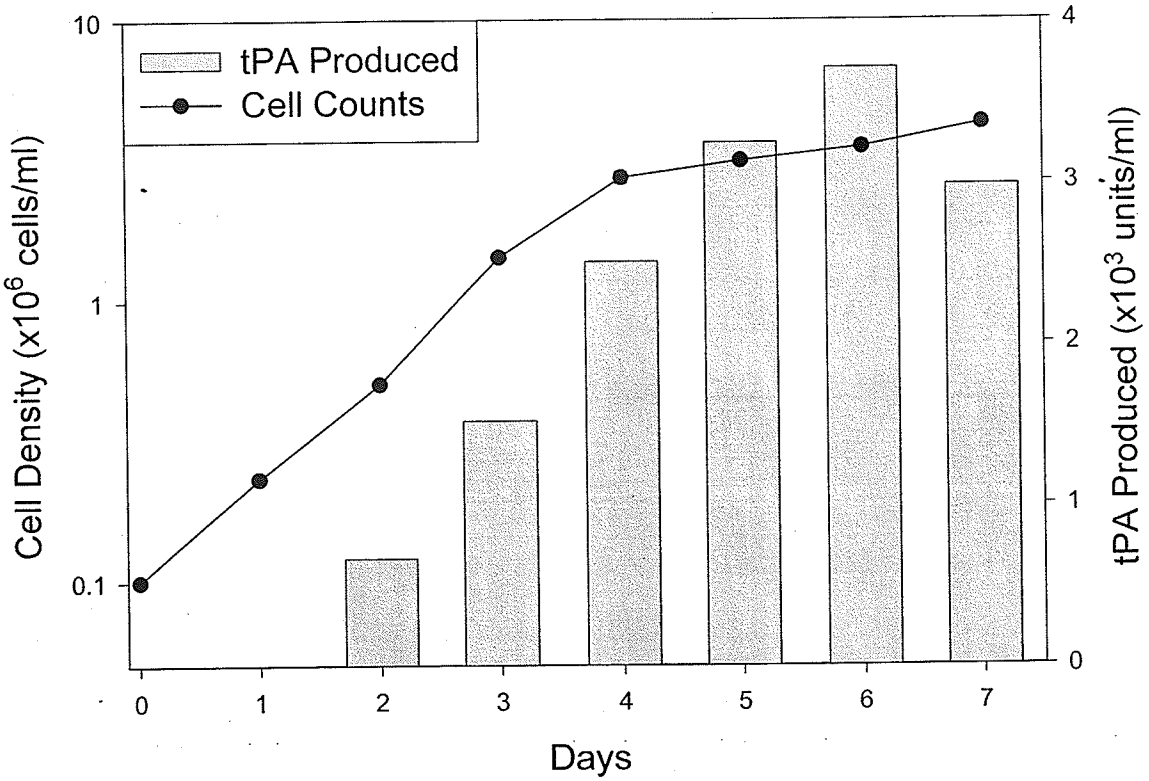


Figure A.3. A representative growth and productivity profile of CHO cells producing recombinant tPA. The tPA was assayed from supernatant samples stored in -70°C until the end of the culture.

evidence of a lower molecular weighted band at approximately 35-40 kDa. However, this band was much lower in concentration compared to the decrease in the 70 kDa band.

This decrease in tPA protein product was also reported by Lin *et al.* (1993) when CHO cells producing tPA exposed to anoxia showed a drop in tPA production. The drop in tPA protein correlated with a 40 % decrease in tPA specific activity due to extensive tPA degradation. Dutton *et al.* (2006) alluded to a relationship between the expression of recombinant protein and the cell cycle phases, where tPA production declined overtime due to a combination of declining secretion and enhanced degradation of extracellular tPA in CHO cell cultures. This degradation of tPA was also found to occur in *Aspergillus niger* producing cell cultures, whereby maximum volumetric t-PA activity was observed at approximately 45 h, at the end of the first phase of batch (Wiebe *et al.* 2001). However, past this time period, the total tPA concentration decreased as well the tPA activity.

The tPA ELISA protocol required optimization to ensure no interference from media components. I completed numerous tests to ensure that the ELISA was sufficient for our samples. As well, there are two tests to detect the levels of tPA within supernatant samples – an ELISA and the Chromozyme assay. The ELISA detects overall tPA levels within a sample whereas the Chromozyme assay detects the biologically active tPA present within the sample. However, in cultures where the cell viability remained high, the ELISA and Chromozyme assay yielded similar tPA titres, and thus it was decided to assay tPA by the ELISA method because it was repeatable.

Temperature of storage was tested with tPA (Figure A.4). Samples of tPA were stored at -20°C, 4°C, 20°C and 37°C for 48 hours prior to being assayed by the Chromozyme assay. Samples stored at -20°C showed no signs of lowered protein activity from the original sample. Samples stored at 4°C and 20°C showed assay values of 90-95 % compared to the original sample. However, samples stored at 37°C overnight assayed at 83 % of the original sample. Thus samples were stored at -80°C for longterm sample storage (3 months to a year) or -20°C for short term sample storage (within 3 months). Storage at these temperatures did not seem to affect measured tPA titres. The samples were stored in small aliquots to prevent multiple thawing of samples.

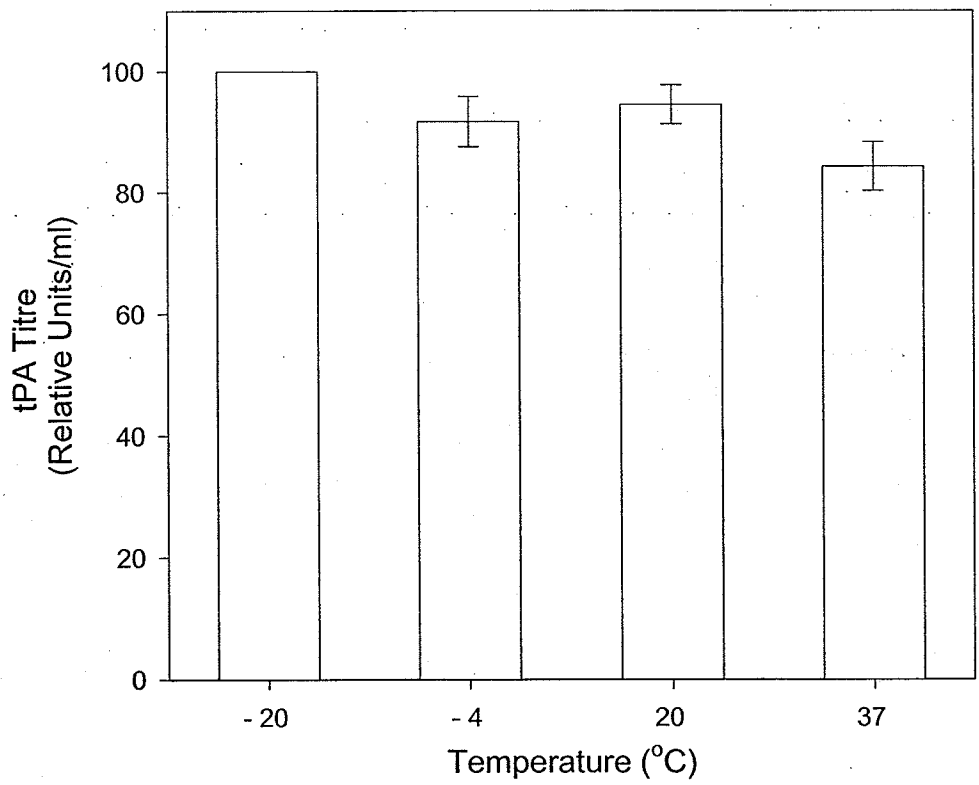


Figure A.4: Effect of storage conditions of tPA supernatant at different temperatures after 48 hours of storage at varying temperatures. The tPA was assayed by the Chromozyme Assay. The values are averages of triplicate samples ± SEM.

Appendix B
Microcarrier Counting Technique

B.1 Introduction

Microcarriers such as Cytopore are composed of a dextran mesh which entraps cells and allows them to grow to a high cell density. A major problem with the growth of these cells in the dextran mesh, is the enumeration of the cells. The separation of intact cells from the porous microcarrier is virtually impossible, and thus different counting methods must be analysed for efficient counting.

The recommended counting method involves the removal of nuclei from Cytopore by addition of a 0.1M citric acid solution with Triton detergent (2 % v/v) with crystal violet solution (Spearman *et al.*, 2005; Yokomizo *et al.*, 2004). However, when looking at high cell densities, it is questionable as to whether the nuclei are being completely removed from the microcarrier.

Thus in this section we investigated the mechanisms of counting, also proving that the crystal violet removal method is sufficient for nuclei removal from these macroporous microcarriers.

B.2 Results

A reliable method of cell counting was required to allow growth in the Cytopore cultures to be monitored. Brightfield photomicrography of cells stained with crystal violet indicates the level of cell colonization within the microcarriers. Figure B.1 shows

the staining of individual microcarriers taken at day 2 and day 4 of a culture and represents approximately 50 and 400 cells/bead respectively. However, the density profile offered by such photomicrographs are only semi-quantitative as an indicator of cell concentration within the microcarriers.

The validation of our counting technique is important to ensure that the nuclei counts obtained within the microcarriers are an accurate measurement of cell growth. The method used in the lab, also recommended by GE Healthcare, involve treatment of cells in the Cytopore microcarriers to 0.1M citric acid, 2 % (v/v) Triton, and 0.1 % crystal violet. The method involves shaking a 1 to 1 ratio of cells to citric acid mix for up to 3 hours and then aspirating through a 25-guage needle 25 times. The method has been deemed to be efficient for removal of CHO cells from the Cytopore pores (Spearman *et al.*, 2005).

However, we wished to investigate the complete removal of cells from the Cytopore. As a result we aspirated microcarriers up to 30 times through a 25-guage needle and counted nuclei release from the samples after each aspiration. In attempt to keep similar shear stresses within the needle for each aspiration, we aspirated independent samples, thereby accounting for the removal of the microcarriers after each sample count.

Figure B.2, represents the stained nuclei removed from the microcarrier. Each aspiration removed nuclei from the microcarrier up to n=30. We considered the nuclei released with 30 aspirations as the maximum number of nuclei possible within the

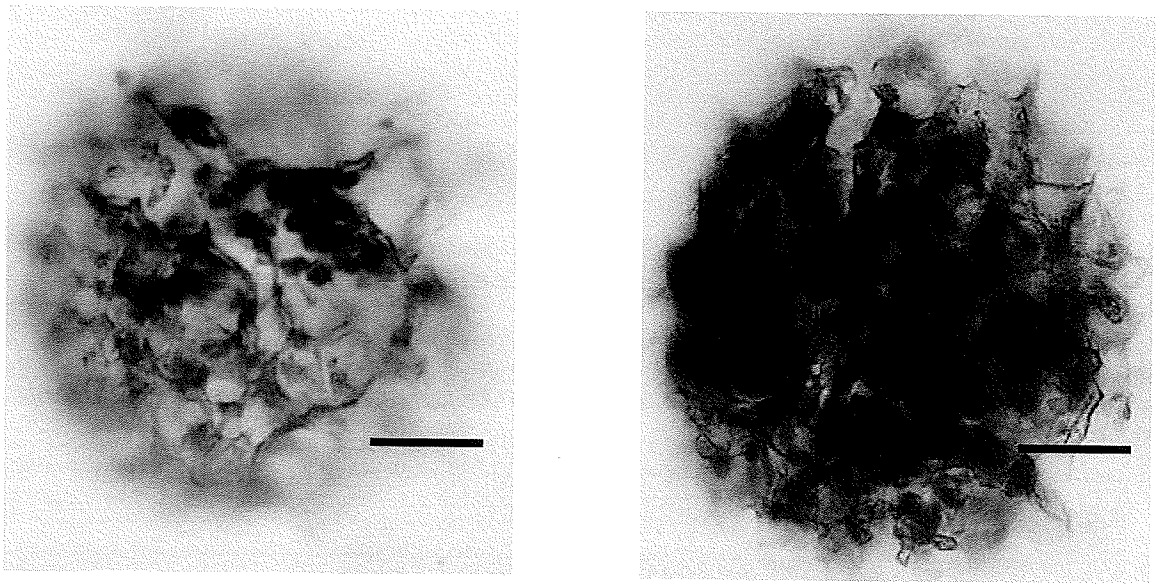


Figure B.1: Brightfield photo micrograph of CHO cells grown in 1.0 mg/mL Cytopore 1. The left micrograph indicates a microcarrier with approximately 50 cells (day 2) and the right image represents a microcarrier with approximately 400 cells (day 4). Microcarriers were stained with crystal violet. Each bar indicates 50 μm.

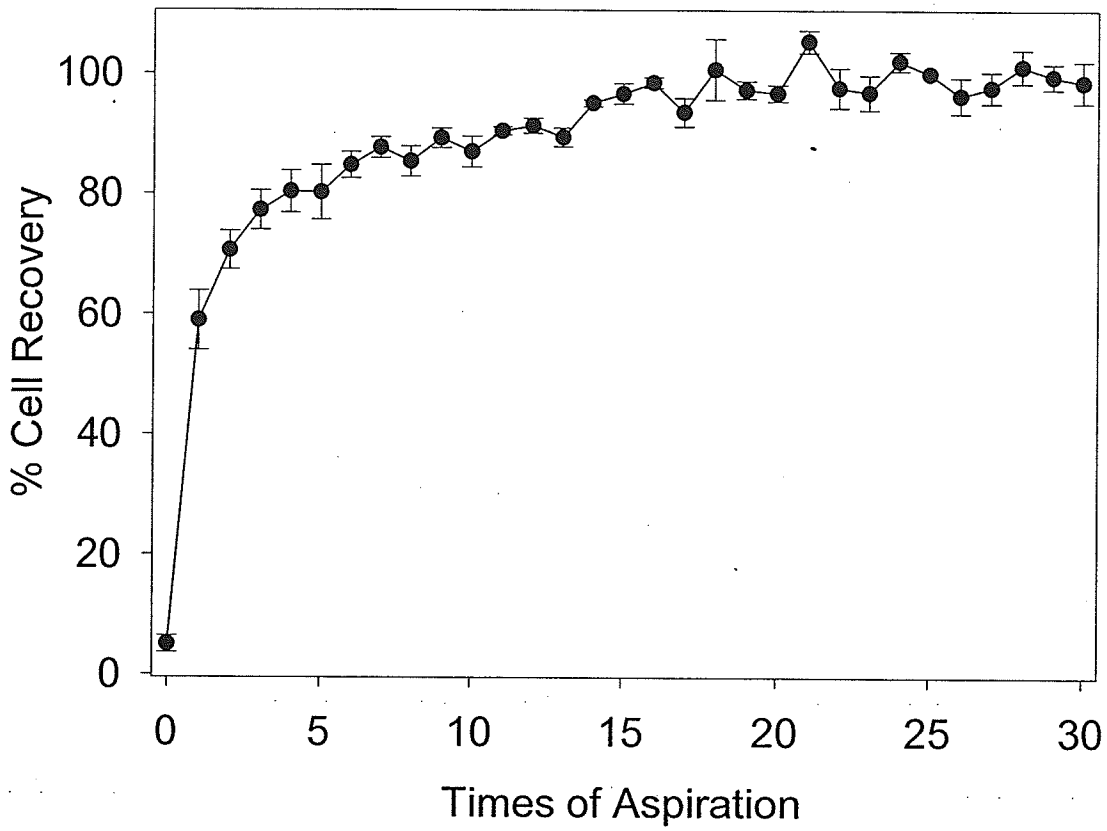


Figure B.2: The cell recovery from microcarriers when aspirating through a 25-guage needle varying times. The cells were treated with a citric acid solution then enumerated by crystal violet nuclei staining. The number of nuclei released at n=30 was considered to be 100 %.

sample, and thus compared the nuclei released with less aspiration to the value at n=30 to obtain the percent released. The graph indicates that as the aspirations increased the nuclei released into the supernatant increases to a maximum at n=20. By comparing the nuclei released into the supernatant we were able to validate the maximum aspirations required to release all nuclei from the dense microcarrier.

Visualization of the microcarriers under light confirmed the removal of nuclei. The microcarriers stained with the crystal violet solution were then washed with PBS to remove excess crystal violet from the microcarriers. The washing of the microcarriers with PBS entailed allowing the microcarriers to settle, then removing excess buffer and adding fresh buffer of the same quantity. The dilution of the buffer allowed us to clearly see the crystal violet taken up by the nuclei, and allowed for the visualization of residual nuclei left behind by the aspiration technique.

The removal of nuclei from the microcarriers was also visualized fluorescently. The cell removal was similarly done, where 0.1 M citric acid and 2 % Triton was added to microcarriers and they were aspirated up to 30 times. TO-PRO-3 was then added to the microcarriers in a 5 μM concentration and visualized (Excitation 640 nm / Emission 690 nm). The nuclei images obtained from the TO-PRO-3 fluorescence were overlayed with Cytopore (Excitation 470 nm/ Emission 525 nm) and shown in Figure B.3 (A-E). This enabled us to completely visualize the remaining nuclei retained on the bead.

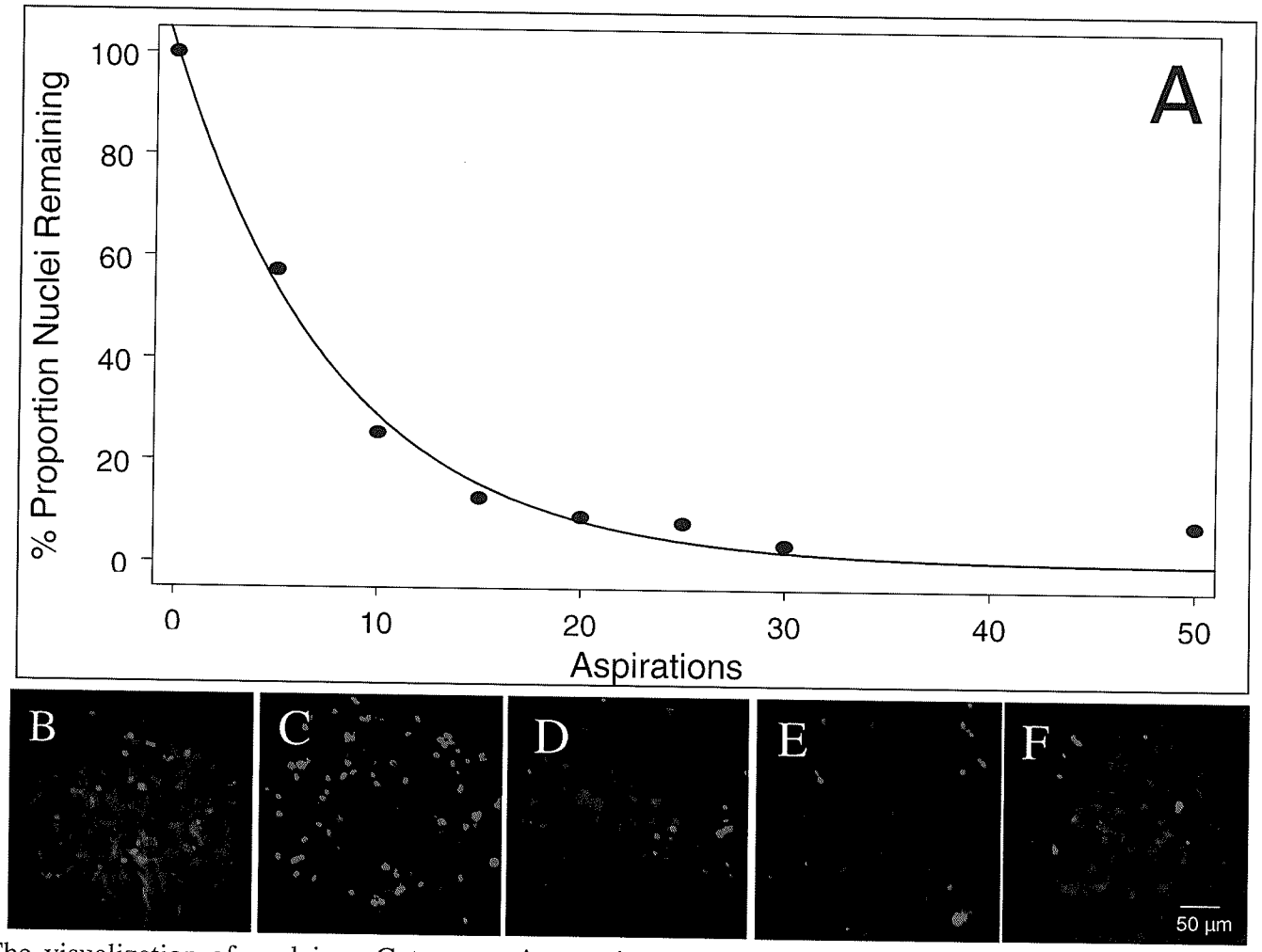


Figure B.3: The visualization of nuclei on Cytopore microcarriers. The nuclei were stained with TO-PRO-3 then overlayed with autofluorescence from Cytopore microcarriers. The integration of the area taken up by nuclei was plotted in the graph (A). Images B through F show aspirations at n=0, n=10, n=20, n=25, and n=30, respectively. The residual nuclei remaining at n=30 was considered to be about 5 % of the initial full microcarrier.

Figure B.3 (B-F) clearly shows the residual nuclei left behind by the counting technique aforementioned. The nuclei left behind on the microcarrier are represented by the darkly stained areas of the microcarrier. In Figure B.3B, we see a microcarrier that has solely been treated to the crystal violet solution with no aspirations. We could see that the nuclei remaining on the microcarrier is high, thus indicating that agitating the microcarriers in the citric acid solution for 30 minutes allows for the removal of just 5 % of nuclei. In Figure B.3F, we have a microcarrier that has been agitated in the crystal violet solution for 30 minutes and aspirated through a 25-guage needle for 30 times. The image clearly shows that there was the removal of 95 % of nuclei by following this method. Figures B.3 C-E show the microcarriers after aspiration n=10 (C), n=20 (D), n=25 (E) times. The integration of area of space taken up by nuclei is shown in Figure B.3 A. The nuclei remaining after aspiration n=20 times, show that n=20 aspirations are sufficient for nuclei removal from the microcarriers. Subsequently, we adopted a step of 25 aspiration cycles in our standard operating protocol in order to ensure the complete removal of stained nuclei from the microcarriers.

The use of Cytopore in cultures leads to a problem in the isolation of cells. As we are unable to isolate cells from the microcarriers, and we thus must use nuclei counts to determine the cell density of the cells. The enumeration of nuclei generally leads to counts higher than the actual cell number due to the presence of binucleated and multinucleated cells and the presence of micronuclei within the cell population. Also there is no way of differentiating the nuclei isolated from live or dead cells. Thus towards

the end of cultures, there is generally up to 2-fold higher cell counts if counted by the crystal violet method.

Figure B.4 shows the counts of a suspension culture by trypan blue (cells) and crystal violet (nuclei) counts. At the beginning of a culture, during the lag phase, the cell and nuclei counts are consistent. However, as the cells enter the exponential phase, the cell and nuclei numbers become varied. In the stationary and death phases, the cells appear to become bi- or multi- nucleated, as well the presence of residual nuclei from lysed and dead cells, such that the nuclei counts increase whereas the cell counts depreciate over time. The increase in the nuclei is consistent with the increase in dead cells in the culture.

The data obtained from the enumeration method of the Cytopore cultures are known to be inconsistent with the live cell counts. However, we must remain consistent when comparing Cytopore and suspension cultures to allow for comparison between the two different types of cultures. Although there are inconsistencies between the ratio of cells and nuclei throughout the life cycle of the cultures, the counting of the nuclei of each culture is important to ensure reasonable comparisons between cultures.

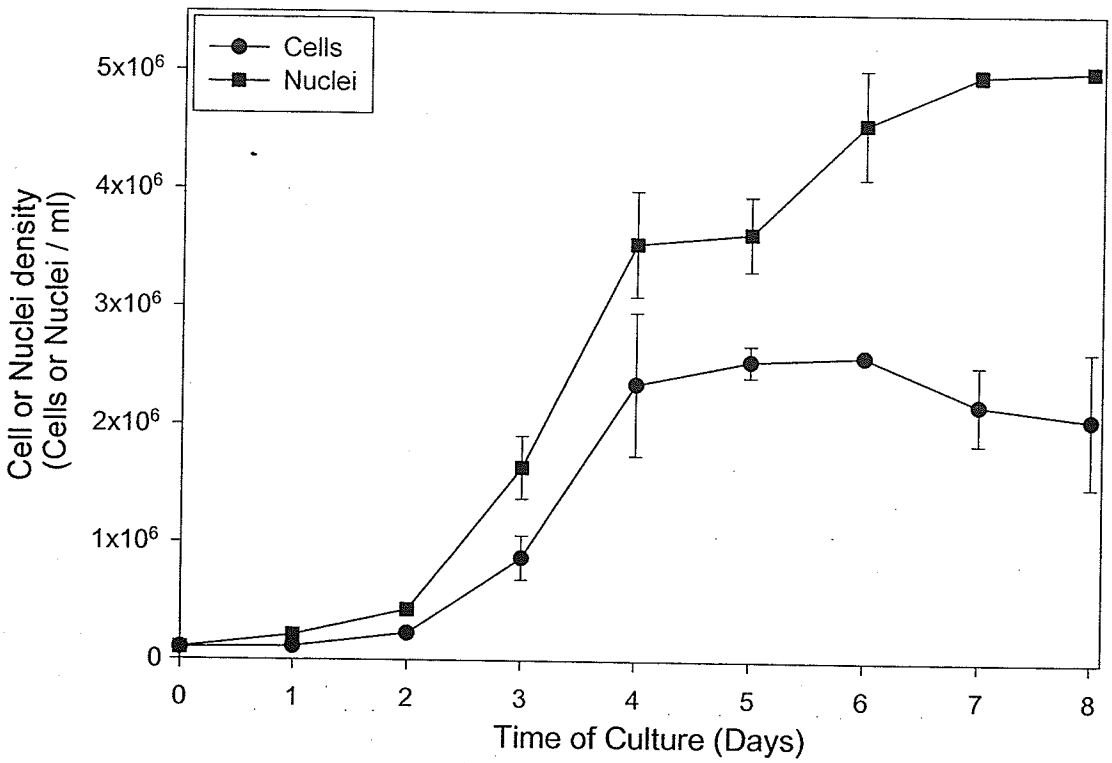


Figure B.4: Comparison of nuclei and cell counts in suspension cultures. The cell density was estimated using the trypan blue exclusion method and the nuclei density by crystal violet staining. The single points are represented an average of n=2.

Appendix C

Growth and Productivity of IFN-beta in biphasic cultures*

C.1 Introduction

Exposure of cultures to low temperature can enhance cell specific productivity (Yoon *et al.* 2003). In the following experiment our objective was to maximize volumetric productivity by combining the beneficial effects of enhanced specific productivity with substantial cell growth. Preliminary work showed that a low temperature of 32°C allowed limited growth following a shift from the standard temperature of 37°C (Rodriguez *et al.* 2005).

C.2 Results

We investigated the effects of a biphasic culture involving a shift to the lower temperature after a period of cell growth at 37 °C (Figure C.1). A temperature shift was induced at days 1, 2, and 3 in an attempt to determine the optimal time to enable a sustainable high cell density and lead to a high product titre. The growth profile of these cultures was compared to those maintained at either 37 °C or 32 °C.

At 37°C the cells grew with a maximum specific growth rate of $0.032h^{-1}$ during the exponential growth phase and reached a maximum density of $2.5x10^6$ cells/mL after 4 days of culture. Cells that were incubated for an equivalent period at 32°C showed negligible growth. After day 12 at this lower temperature the population increased to just over $1.3x10^5$ cells/mL. Reducing the culture temperature to 32°C after 1, 2 or 3 days at

* Experiments of this Appendix were completed by K. Sunley. The contents were included in a paper - Tharmalingam, T., Sunley, K., and Butler, M. High yields of monomeric recombinant beta-interferon from macroporous microcarrier cultures under hypothermic conditions. *Biotech. Prog.* **2008**, 24(4):832-838.

37°C resulted in intermediate cell yields. Analysis of IFN-beta showed a significantly higher production at 32°C from the day 2 temperature culture with a yield of 14.4 x10^6 units/mL (Table C.1).

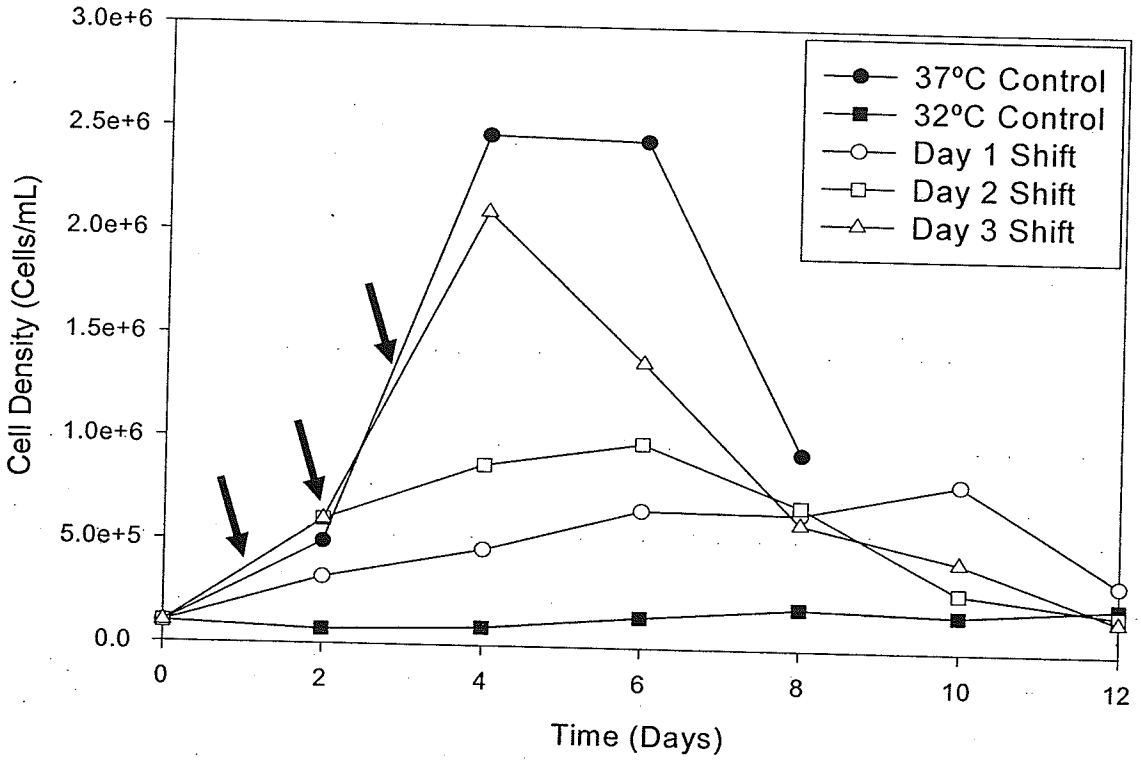


Figure C.1: Growth profile of temperature-shifted cultures (○ ;□; Δ), 37°C (●) and 32°C (■) cultures. The temperature-shifted cultures were allowed to grow at 37°C until the temperature was shifted to 32°C. The arrows represent the day of shift for each corresponding plot: day 1 (○), day 2 (□), and day 3 (Δ).

Table C.1 Volumetric production of IFN-beta from CHO cells grown at 37°C and in biphasic (37°C to 32°C) cultures.

Culture Condition	Temperature (°C)	Shifted Day	Native Protein (units/mL)	Denatured Protein (units/mL)	Aggregation (%)
Monophasic	37	N/A	1.81×10^6	4.64×10^6	61
	32	N/A	1.39×10^6	1.98×10^6	30
Biphasic	37-32	Day 1	7.08×10^6	8.14×10^6	13
	37-32	Day 2	11.38×10^6	14.4×10^6	21
	37-32	Day 3	12.8×10^6	12.8×10^6	*

* Refers to negligible aggregation