OPTIMISATION OF RETROVIRAL PRODUCTION SYSTEMS FOR GENE THERAPY APPLICATIONS

by

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Abstract

Retroviral vectors are a promising tool for gene therapy. However, there are two major problems to overcome if a viable commercial production process is to be established. These are the instability of virus particles and the low virus titres.

The characteristics of the producer cells were determined in batch culture, semi-continuous culture and semi-continuous culture at 32°C. Additionally, cell attachment, growth and virus production on various macroporous microcarriers was assessed under static and stirred conditions.

Alternative strategies for the cultivation of cells were also investigated. These included spinner basket, packed bed and spinner flask cultures with semi-continuous feeding and packed bed, fixed bed, fluidised bed and stirred tank cultures with continuous perfusion of culture medium. Of these the fixed bed bioreactor had the highest cell specific productivity and was capable of running for 28 days. The fluidised bed bioreactor had the highest reactor productivity, due to the higher cell number.

Optimisation of culture medium was performed with regard to serum concentration. The greatest production was observed at an initial serum concentration of 2.5% (v/v).

The findings in this thesis will assist the development of an efficient method for the production of clinical grade retroviral vectors for gene therapy applications.

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Finally, I would like to express my appreciation to God, without whom none of this would have been possible.

Of making many books there is no end, and much study wearies the body.

Now all has been heard;
here is the conclusion of the matter:
Fear God and keep his commandments,
for this is the whole duty of man.

Ecclesiastes 12:12-13

Contents

Chapter 1: Introduction

1.1 Gene Therapy

Gene therapy is one of the most significant breakthroughs in modern medicine. In principle, heterologous genetic information is inserted into damaged or defective tissue or cells, enabling them to function correctly and eliminate the disease symptoms. Gene therapy was initially developed to treat inherited monogenic disorders, such as cystic fibrosis and muscular dystrophy (Andreadis et al. 1999; Cruz et al. 2000; Johnson 2001) but it has the potential to treat several human diseases. These include cardiovascular disease, infectious diseases such as HIV, Herpes and Hepatitis, and degenerative disorders, particularly those that are neurological (Hiltunen *et al.* 2002; Islam *et al.* 1997; Kim *et al.* 2000; Lauret *et al.* 1993; Mautino and Morgan 2002; Palu *et al.* 1999).

The majority of clinical gene therapy trials are for the treatment of cancer (Marchisone et al. 2000). While cancer may be considered a genetic disorder due to the number of mutations in evolving clones of tumours, attempts to correct these mutations by gene therapy would be impossible in most cases, due to the huge range and complexity of mutations (McTaggart and Al Rubeai 2002). Instead, gene therapy is used to deliver cancer killing agents to the tumour cells.

Several strategies have been conceived for the treatment of diseases with an acquired genetic component. Thus, the original concept of gene therapy has evolved to include:

1. The delivery of genes able to kill the targeted cell in a controlled manner (suicide-gene therapy).

2. The delivery of transdominant functions or sequences, like anti-sense molecules and ribosomes, capable of switching off a functional gene (suppressive gene therapy).
3. The delivery of immune functions capable of inducing the immune system to destroy cells expressing specific determinants (immune gene therapy). (Palu *et al.* 1999).

1.2 Gene delivery

The key to success for any gene therapy strategy is having a vector able to serve as a safe and efficient gene delivery vehicle (Friedmann 1997). Various kinds of synthetic vectors are currently being investigated as an alternative to viral vectors (Navarro et al. 1998). They can be divided into two sub-groups; cationic lipids and molecular conjugates (Gao and Huang 1995; Michael and Curiel 1994). Simple mixing in vitro of the synthetic reagent with plasmid DNA leads to the spontaneous formation of DNA/vector aggregates via a self-assembly process. For efficient gene transfer these must survive in the extracellular medium and reach the target cells. Once the vectors have reached the target cell several steps, including binding, internalisation, endosomal release, uncoating, nuclear escape and expression, are required to achieve efficient gene delivery and expression. While non-viral methods are generally considered to be safer than viral transduction, they are less efficient, and no commercial processes have been developed to meet the potential market demand (Theodossiou *et al.* 1999). Viruses, however, have complex and precise structural features, adjusted through natural evolution for efficient transfection of host cells (Navarro *et al.* 1998).

A number of virus types are currently being investigated for use as gene delivery vectors. These include retroviruses, adenoviruses, adeno-associated viruses, poxviruses, rhabdoviruses, parviruses and alpha viruses (Palu *et al.* 1999). It is unlikely that any one of these vectors will emerge as a suitable vector for all applications. Instead, a range of vectors will be necessary to fulfil the objectives of each treatment (McTaggart and Al Rubeai 2002). Although vectors derived from adenovirus, adeno-associated virus and herpes virus are receiving more attention in the field of gene therapy (Benihoud *et al.* 1999; Parasrampuria 1998; Vos 1995), recombinant retroviruses are currently used in the majority of clinical trials (Kang S-H. *et al.* 2000; Wu and Ataai 2000; Wu *et al.* 2002).

1.3 The use of retroviral vectors in gene therapy

1.3.1 Retroviruses

Retroviruses have a number of advantages as gene therapy vectors and they are currently the only vectors capable of efficiently introducing genes into haemopoietic stem cells (Onions and Lees 1994). They are enveloped viruses that contain two identical copies of single stranded RNA, typically 7-10 kilo base pairs in length, as their viral genome. The diameter of retrovirus particles has been reported to be 90-147nm (Kafri et al. 1997). The structure of a retrovirus is illustrated in Figure 1.3.1. During the life cycle, the ssRNA is reverse transcribed to yield double stranded DNA that integrates into the host genome and is expressed over extended periods. As a result infected cells continuously produce virus particles with no apparent damage to the host cell (Smith 1995). However, for many types of retroviruses, mitosis and the breakdown of the nuclear envelope are required for the viral DNA to be transported to the nucleus. Thus, successful infection can only occur in actively dividing cells. A

notable exception to this are the lentiviruses, which include HIV, and can infect non-dividing cells (Lewis and Emerman 1994), a crucial asset in cells lines such as neurons and haematopoietic stem cells (Kafri *et al.* 1999; Klages *et al.* 2000). The permanent integration of the virus into the host cell genome allows the potential for stable gene expression, making this an attractive option for the treatment of genetic disorders. In reality, though, the frequent loss of gene expression *in vivo* has meant this has been difficult to achieve. An additional problem associated with retroviral vectors is the random insertion of the viral DNA into the host genome. This can lead to insertional mutagenesis or the activation of oncogenes. Random insertion can also result in varying levels of therapeutic expression, as this is reliant on the site of integration (Gordon and Anderson 1994).

Retroviruses can be divided into three subfamilies, Oncovirinae, Lentivirinae, and Spumavirinae, according to their pathogenicity and are further classified by their virion structure, cell receptors and oncogenicity (Kim *et al.* 2000). The structure of the virus particle is extremely simple, comprising of three genes encoding gag, the core proteins; pol, the reverse transcriptase; and env, the viral envelope protein. At the ends of the RNA genome are the long terminal repeats (LTRs) which include promoter and enhancer activities and sequences involved in integration. The genome also includes a sequence required for packaging, termed psi (Ψ), a tRNA binding site, and several additional sequences involved in reverse transcriptase.

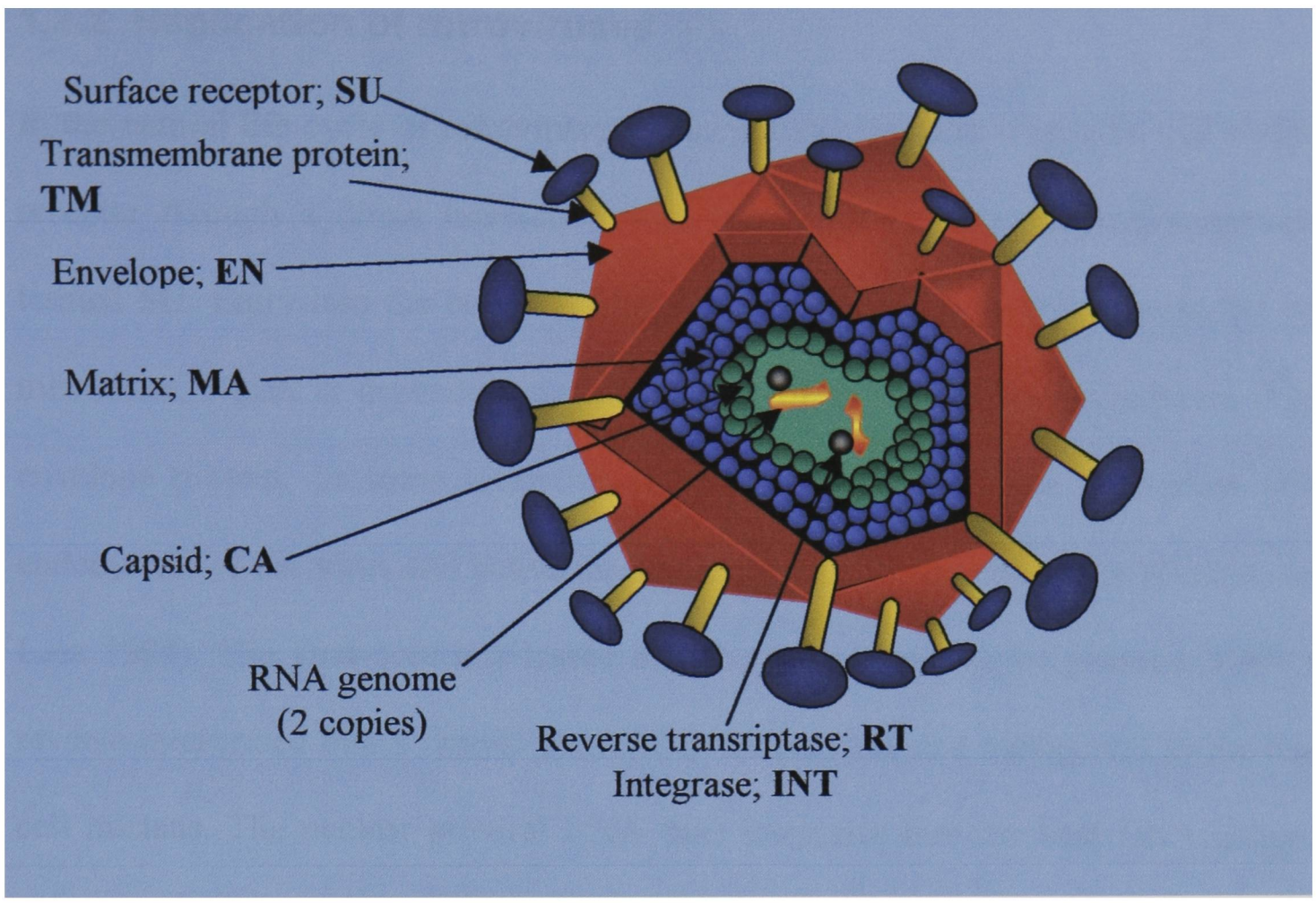


Figure 1.3.1

Three-dimensional representation of a retrovirus particle.

1.3.2 Replication of retroviruses

In the natural life cycle of retroviruses, virus particles bind to a specific cell surface receptor through a direct interaction with the major viral envelope glycoprotein, termed SU. Entry into the cell is mediated by fusion of the envelope with the cell membrane, which is dependent on the activity of the minor transmembrane (TM) envelope protein. In some retrovirus infections this fusion may take place after endocytosis of the virus and activation in the low pH of the endosome (Onions and Lees 1994). The viral core is released into the cytoplasm and the genomic RNA is reverse transcribed into a double stranded DNA provirus and transported to the host cell nucleus. The nuclear proviral DNA then integrates into the host DNA using a virally encoded integrase (INT). Proviral DNA is transcribed by the cell, producing RNAs encoding gag, pol, and env proteins, allowing for the packaging of the full length unspliced viral RNA containing the Ψ packaging site. Following virus assembly, the gag polyprotein is proteolytically cleaved by a viral protease into smaller polypeptides that include matrix (MA), capsid (CA), and nuleocapsid (NC). At least one of these binds to the Ψ site to facilitate packaging into the membrane associated virion. The assembled particles are released from the cell by budding from the env coated cell membrane. An important feature of retroviruses is that infected cells stably produce viruses for the duration of their life span without their growth properties being affected (Kim *et al.* 2000). The life cycle of a wild type retrovirus is illustrated in Figure 1.3.2.

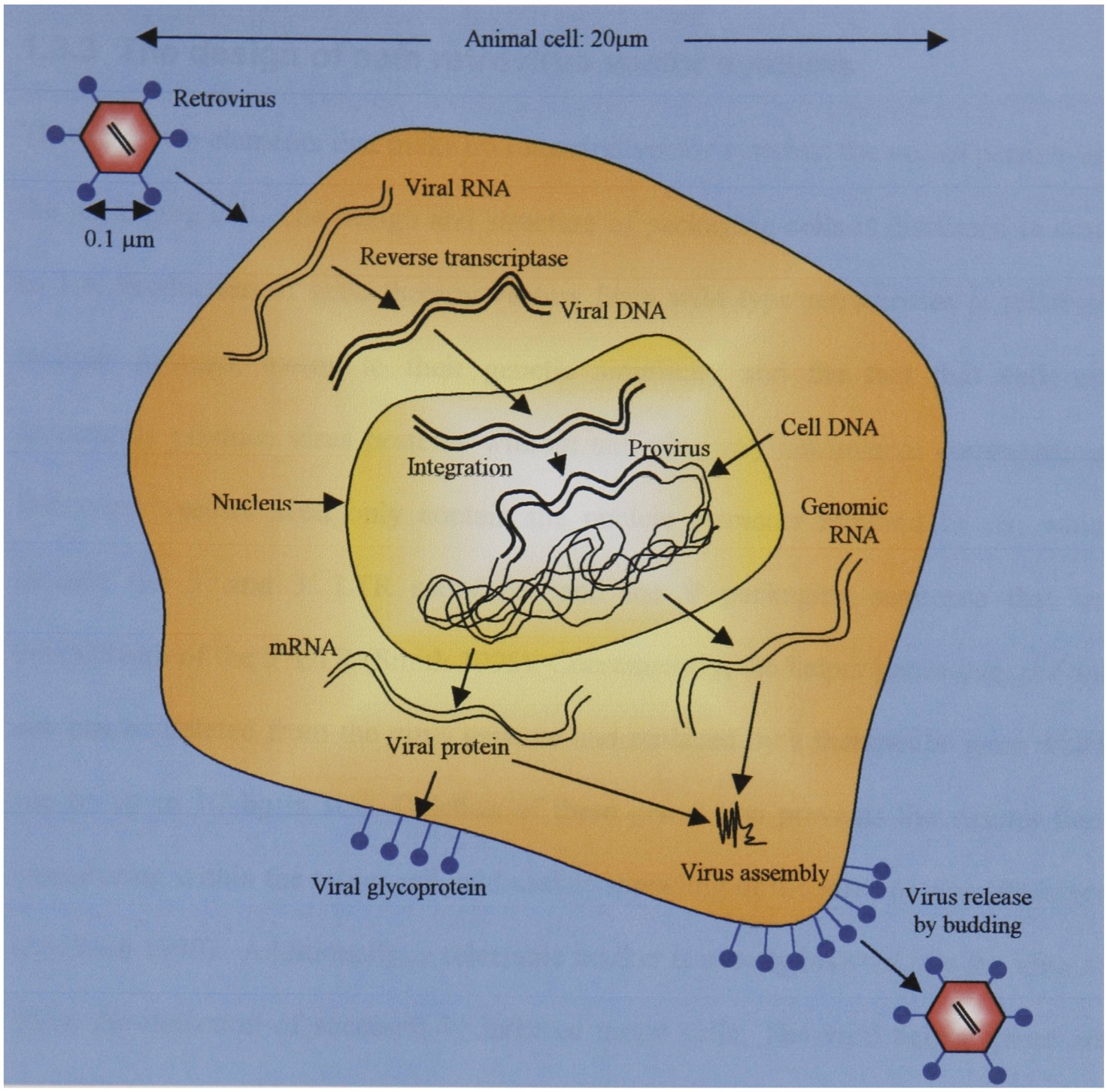


Figure 1.3.2

The natural life cycle of a retrovirus (not to scale).

1.3.3 The design of safe retrovirus vector systems

There are two elements that make up retroviral vector systems; the vector particle and the packaging cell. The design and structure of packaging cells is discussed in detail in 1.4. Production of gene therapy vectors from wild type retroviruses is relatively straight forward, owing to their genetic simplicity and the fact that cells can incessantly produce virus proteins without suffering any detrimental consequences. Retroviral vectors need only contain the nucleic elements required in *cis*, which include the 5′ and 3′ LTR sequences and the Ψ packaging sequence that lies downstream of the 5′ LTR (Smith 1995). Consequently, the helper genes *gag, pol* and *env* can be deleted from the virus genome and replaced by a therapeutic gene which can be up to 10kbp in size. Deletion of these genes also prevents the viruses from reproducing within the target cell, and avoids spreading of the virus *in vivo* (Pedersen and Duch 1999). Additionally, a selectable marker is usually inserted into the virus to allow the detection of successfully infected target cells. The viral helper genes are contained on plasmids present in the packaging cell line.

A problem encountered in first-generation vectors was a significantly reduced virus titre when compared to wild type viruses. To overcome this, modifications have been made in the packaging signal of currently used vectors, whereby the N-terminal *gag* sequences are inserted adjacent to the packaging sequence (called the Ψ+ region)(Adam and Miller 1988; Armentano *et al.* 1987). This has led to vastly improved virus titres, such that levels are similar to those of the wild type virus, providing the Ψ and *gag* sequences are kept together (Morgenstern and Land 1990). The potential expression of the initiation codon for gag synthesis rather than the gene of interest can be prevented by mutation of the AUG sequence or insertion of a

downstream termination codon (Bender et al. 1987). To date, more than 100 genes have been expressed using the retroviral vector system (Morgan *et al.* 1993).

1.4 Packaging cell lines

The second element of a retroviral vector system is the packaging cell line. These cells contain the virus helper genes (*gag, pol* and *env*) that are absent from the vector genome. Producer cell lines are made by inserting the vector, which has had these genes replaced by a therapeutic gene, into the packaging cell on a separate plasmid (Pear et al. 1993). Hence, the structural and functional proteins required for the synthesis of new virus particles are produced by the cell. Recombinant viruses are released from the cell in a similar fashion to wild type viruses. Packaging cells are unable to produce their own virus particles as the Ψ packaging sequence and the LTRs are only contained on the vector plasmid (Robbins *et al.* 1998). The mechanism of producing retroviral vectors from packaging cell lines is illustrated in Figure 1.4.1. Packaging and producer cell lines are able to continuously release vector particles, making them well suited to large-scale production systems. This gives them a significant advantage over transient transfection systems, such as baculovirus or adenovirus systems, where generation of large volumes of characterised supernatants is difficult (Rigg et al. 1996).

Many technological improvements are still required for vector design and production. These improvements include higher virus titres, human compliment-resistant vectors, and minimized potential for generation of replication-competent retroviruses (RCR) (Sheridan et al. 2000). Theoretical predictions have suggested that 10^8 virions ml^{-1} of infusion product would be required to treat a tumour, based on the supposition that a typical tumour contains 10^9 cells g^{-1} and a diseased organ may contain 100g of tumour. This figure also assumes that the transduction efficiency would be 100%, no particles

are lost by compliment inactivation and that infection is not limited by cell replication. Therefore, it was recognised that the actual viral dose may be 10 -1000 times greater (Braas *et al.* 1996; Lyddiat and O'Sullivan 1998). It has subsequently been shown empirically that titres >5 x 10^7 virions ml^{-1} of infusion product can efficiently obtain *in vivo* gene transfer in animal models of haemophilia, arthritis, cancer, chronic HBV infection, cystic fibrosis and other diseases (Ghivizzani et al. 1997; Karavodin et al. 1998; Nemunaitis et al. 1999; Sallberg et al. 1998; VandenDriessche et al. 1999; Wang et al. 1998). Therefore, much attention has been focused on the development of high titre packaging cell lines. Calcium phosphate-mediated transfection was shown to be a reliable method for introducing vector genes into packaging cells. The resulting producer cells were capable of producing >10^6 virions ml^{-1} (Pear *et al.* 1993). An alternative method for generation of producer cells is by retroviral infection. In comparison, this procedure was able to yield up to 5 x 10^7 virions ml^{-1} more than transfected clones. The titre was increased by multiple rounds of infection (the "ping-pong" strategy) through long-term incubation of producer cells with ecotropic virus vectors, resulting in a 20-fold increase in vector titre (Kim *et al.* 1998). However, the ping-pong method suffers from the increased probability of recombination between retroviral components, leading to RCR (Muenchau *et al.* 1990). Sheridon *et al.* (2000) have shown that improved viral titres can be obtained by increasing the multiplicity of transduction, thus introducing multiple copies of the provector within vector producing lines. This has only been possible through the development of packaging cells with reduced homology between viral vector and packaging components.

A major drawback to *in vivo* gene delivery is the inactivation of virus particles by human serum. The virus decay rate *in vivo* correlates with their *in vitro* resistance to serum complement. The sensitivity of retroviral vectors to human serum is dependent

on the species of the packaging cell line and vectors produced from murine cell lines have been shown to have a half-life <1 minute following intravenous administration (Cornetta *et al.* 1990). Viruses produced from non-primate cells are sensitive to human serum because they bear Gal(α1-3)galactosyl sugar on their envelope, which activates natural antibodies present in human serum (Takeuchi *et al.* 1996). Hence, human cell lines that do not express Gal, such as HT 1080 and TE671, have been used as packaging cells (Cosset *et al.* 1995; Higashikawa and Chang 2001). In addition to the packaging cell type, the type of envelope protein can also influence complement sensitivity of MLV vectors (Takeuchi *et al.* 1994).

Aside from the problems associated with the levels of expression and complement inactivation, there are concerns over the safety of using retroviral vectors, especially lentiviruses, which include HIV-1. Recombination between the vector and the helper genes can lead to the generation of wild type replication competent virus. The demonstration that producer cells do not release RCR is the most important criterion in using retroviral vectors (Onions and Lees 1994). The probability of producing RCR has been reduced by using separate plasmids for the *gag-pol* and *env* genomes, and by using genes from viruses with different host species. The possibility of formation of RCR can be further reduced by decreasing the viral sequences in the helper constructs (Cosset *et al.* 1995). However, RCR can still be produced through recombination with endogenous retroviral sequences. Early packaging cell lines were derived from NIH/3T3 cells, which express endogenous MLV virus sequences, and participate in recombination to form RCR, particularly in mass culture during large-scale clinical vector production (Rigg *et al.* 1996). For that reason, cell lines are now screened for endogenous viral sequences before being developed as packaging cells. An example of a cell line successfully created in this way is the ProPak packaging cell, which has

been derived from MLV-free HEK293 cells. Using a vector known to reproducibly give rise to RCR in PA317 cells, ProPak cells were shown to be RCR-free using conditions favourable to RCR formation e.g. the ping-pong method (Forestell et al. 1997;Forestell et al. 1998).

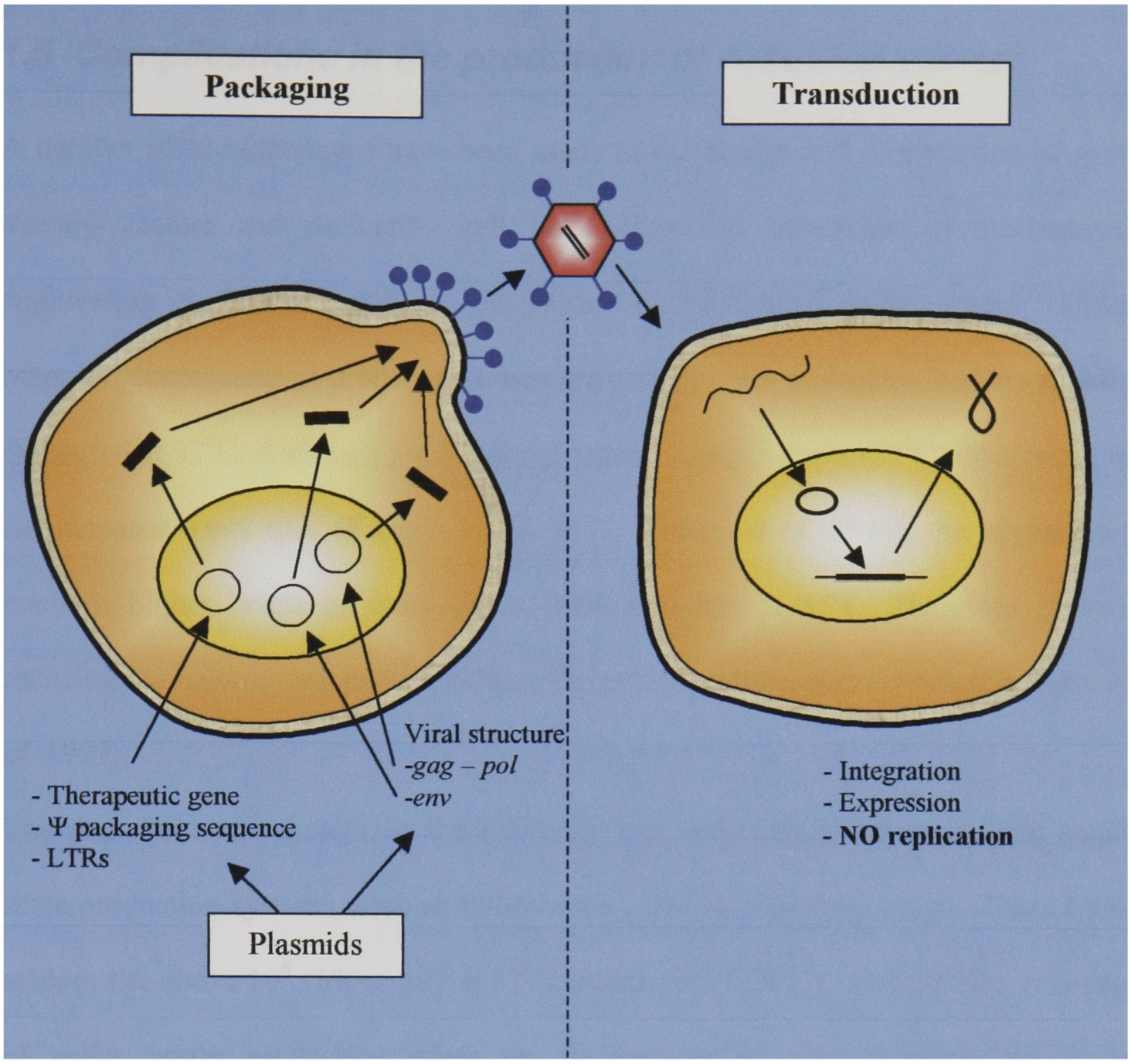


Figure 1.4.1

The principle mechanism of packaging cell lines and engineered retroviral vectors for gene therapy.

1.5 Complications in the production of retroviral vectors

A number of breakthroughs have been made in the design and construction of gene therapy vectors and packaging cell lines. However, a number of biochemical engineering challenges remain in the production of clinical grade vectors. Unlike other biopharmaceutical products, viruses are particles, not molecules, and are rapidly denatured at 37°C. Although production at reduced temperatures (32°C) does result in an increased virus titre (Kaptein *et al.* 1997; Kotani *et al.* 1994), the supernatant contains higher levels of transduction inhibitors than at 37°C. Therefore, simply reducing the culture temperature reduces the potency of the vector stock (Le Doux *et al.* 1999).

The main problem, though, is the relatively low vector titres achieved from small scale production systems, such as roller bottles, that are currently in use. These titres seldom rise above 10^6 virions ml^{-1} at 37°C (Merten *et al.* 2001). Additionally, scale-up of roller bottle production relies on an increase in unit numbers, which is uneconomical due to the extra space required and the added labour intensiveness. Therefore, there is a need to transpose vector production to a bioreactor system with an emphasis on process scale-up and intensification (McTaggart *et al.* 1999).

1.6 Overview of biopharmaceutical production systems

The production of biologics is the central core of animal cell biotechnology. It was estimated that the direct and indirect activities of the biotechnology industry contributed $47billion in revenues to the US economy in 1999 (Ernst & Young 2000). Immobilisation technologies have allowed for the production of large biomasses, which forms the substrate from which a wide range of products can be produced, as listed in Table 1.6.1. Animal cells are used for the production of these products to

allow for the correct post-translational processing, including glycosylation, the formation of disulfide-bond, γ-carboxylation and other modifications to occur, which cannot be achieved using other eukaryotic or bacterial cells. However, the relatively low biomass productivity compared to bacterial cultures and the low product expression rate has meant it has become necessary to scale-up their production in order to meet the high demand for these products. Many biologics, such as viral vaccines, are produced from anchorage dependent cell lines. Although developmental work can be done in monolayer cultures, using either tissue culture flasks or roller bottles, it is not a desirable method for large scale manufacture. This is because scale-up is achieved by increasing the unit number of either flasks or bottles, which is extremely labour intensive and susceptible to contamination (Butler 1987). Modern facilities have overcome these problems by the use of expensive robots; however, it is still not possible to control environmental conditions such as pH or dissolved oxygen levels (Hu and Peshwa 1991). The most successful method for the scale-up of anchorage-dependent cells has been the use of microcarriers, first reported by (van Wezel 1967), which provide a high surface area to volume ratio and can be used in traditional stirred tank bioreactors.

The use of suspension cell lines, such as hybridomas, for the production of monoclonal antibodies has been widely used. Scale-up of these systems is relatively simple and the maximum cell densities achieved in fed-batch cultures is approximately 2×10^7 cells ml^{-1}. Therefore, in order to produce the required amount of product high volume bioreactors are necessary. Aside from this, the simple design and operation, along with flexibility and ease of licensing, these systems are the most popular for manufacturing. However, the immobilisation of cells allows for far greater cell densities to be achieved, whilst retaining these features. It also allows for long-

term perfusion cultures which are economically advantageous as time is saved in turnaround and seed build-up, and because of the higher densities it can reduce the capital equipment and plant costs as smaller vessels can be used (50-100 litres are equivalent to 4000 litres for suspension cultures). Immobilised cell cultures can also enhance cell specific productivity, thus giving even greater savings (Griffiths 1992). When developing a production process it is desirable to avoid nutrient limitation and accumulation of toxic metabolites such as lactate and ammonia. This problem can be overcome by the use of medium perfusion. While spin filters have offered limited success, immobilisation has been adopted as the best method (Griffiths 1992).

The immobilization of cells for the production of biologics can be divided into three main areas. The first is cell retention within a compartment, allowing the free passage of medium. Examples of these systems include hollow-fibre and ceramic matrix bioreactors. While it is possible to achieve a 50-fold to 100-fold increase in unit cell biomass they are difficult to scale-up and are limited to 1-2 litres. The second method is cell encapsulation. This technique uses alginate or agarose beads to entrap the cells and allows the product to diffuse into the culture medium. Finally, the most common system is cell entrapment which uses macroporous microcarriers as a cell immobilisation substrate. The advantage of encapsulation and entrapment is that these processes allow cells to be grown in conventional bioreactors and the potential scale-up of these systems is far greater than with cell retention devices (Griffiths 1988).

Table 1.6.1

Biopharmaceutical products produced from animal cells

Viral Vaccines	RotaShield™, RabAvert™, VAQTA™
Antibodies	Monoclonal antibodies e.g. Capromab pendetide/ProstaScint™
Interferons	Human IFN-γ
Enzymes	Fibrinolytic
Whole cells	Autologous cultured chondrocytes
Insecticides	Insect viruses
Immunoregulators	Human Interleukin-2
Hormones	Insulin
Growth Factors	Epidermal growth factor (EGF), nerve growth factor (NGF)

(Chu and Robinson 2001; Griffiths 1985; Hu *et al.* 2000; Kratje and Wagner 1992; Wang *et al.* 2002)

1.7 Cell retention

As the potential of animal cells for the production of biologics became apparent, many investigators looked to develop new bioreactors in order to overcome the problems of hydrodynamic forces caused by mechanical agitation and bubble aeration that were believed to exist in the traditional stirred microcarrier or suspension cultures. One system that was developed by Knazek *et al.* (1972) was the use of ultrafiltration capillary fibres. In this bioreactor cells attach to the outer surface of semi-permeable fibres, growing in the extra-capillary space (ECS) while medium is circulated through the inter-capillary space (ICS) or lumen. Nutrients are able to diffuse through the fibres, usually made of cellulose acetate, while toxic metabolites diffuse out of the ECS and are carried away from the cells. A unit consists of thousands of fibres housed in a cylinder. A similar device is the ceramic matrix bioreactor. This is a cylinder of porous ceramic with square channels passing through the cylinder. Cells are inoculated into the channels and either attach to the surface or are immobilised in the ceramic pores. Medium is passed through the channels to provide nutrients and remove metabolites. In a ceramic system cells are directly bathed in the recirculating medium, whereas in a hollow-fibre bioreactor cells are only exposed to a slow stream of permeate (Hu and Peshwa 1991). The product accumulates within the ECS and is harvested intermittently.

There are numerous advantages to using hollow-fibre bioreactors (HFBR) and they have received much attention for the commercial production of monoclonal antibodies and recombinant proteins (Kessler *et al.* 1997; Thelwell and Brindle 1999; Williams *et al.* 1997). The main advantage of HFBRs is the high cell densities that can be achieved. Numerous studies have reported near tissue densities of $>10^8$ cells ml^{-1} (Kessler *et al.* 1997; Koska *et al.* 1997). These high densities in turn lead to increased

production and concentration of products and enhance the specific productivity in a relatively small bioreactor (Fassnacht and Portner 1999; Glacken *et al.* 1983; Knight 1989). Tzianbos and Smith (1995) reported that a flat HFBR was used to produce monoclonal antibodies from problematic cell lines and yielded a 200-300 fold increase in the concentration of antibody harvested when compared to levels obtained in static culture. Furthermore, it was possible to maintain antibody production over a two month period, demonstrating the long-term potential for HFBRs. This has been verified in studies conducted by Kessler *et al.* (1997) who consistently produced a MAb for 85 days and Fassnacht *et al.* (1999) that produced antibody for a 6 week period from two different murine hybridoma cell lines. Marx (1998) tested 31 hybridoma cell lines of murine, human, and rat origin and found that the average culture times were 56 days, 84 days and 67 days, respectively, demonstrating that long term cultivation and production are possible in HFBR.

A further advantage of HFBRs is the total amount of serum used in production can be lower than that used in other types of bioreactor. The high molecular weight of serum makes it difficult to diffuse into the ECS and therefore only low concentrations are required in the culture medium, and in some cases it can be totally removed. Serum is the most expensive component in the production of biologics and may cause complications in the downstream processing of products. Therefore, removal of serum from any process is highly desirable not only for economical reasons but also removal of components of animal origin in the manufacture of therapeutic and diagnostic products are favoured by regulatory authorities. It has been shown that a reduction in serum concentration to 2.5% can also lead to an increase in antibody production in hybridoma cell lines, although productivity decreases at concentrations ≤1% (Kessler *et al.* 1997;Tzianbos and Smith 1995).

Whilst HFBRs offer a number of advantages, these systems are associated with several disadvantages including poor cell viability, large diffusional gradients which limit scale-up and effect product quality, and difficulties in culturing anchorage dependent cell lines. As medium enters the lumen it encounters a positive transmembrane pressure and is able to permeate into the ECS. As this pressure decreases along the length of the fibre, it becomes negative towards the outlet, causing medium to flow back into the lumen ICS. This phenomenon is called Starling flow (Starling 1896). This results in a higher cell density at the inlet end compared to the outlet or, with suspension cells, a packed cell mass accumulating at the outlet end (Griffiths 1988). The resulting presence of large numbers of dead cells limits culture performance in two ways. Firstly, there is a need for high rates of cell proliferation to replace lost cells, which will reduce the specific productivity (Al Rubeai *et al.* 1992). Secondly, the release of proteases, DNA and other cellular components will lead to product degradation and will complicate downstream processing (Fassnacht *et al.* 1999). Aside from the adverse effect on cell growth, these gradients also affect the quality of secreted products. Glucose concentration and culture pH have both been shown to affect the glycosylation patterns of secreted proteins (Borys *et al.* 1993; Hayter *et al.* 1992). The delivery of oxygen is a particular problem and is considered to be the main limiting factor in cell growth and protein production (Williams *et al.* 1997). In many HFBR systems high molecular weight proteins, either introduced with the cell inoculum (e.g. serum) or produced by the cells (e.g. protein products) are unable to permeate through the fibres and accumulate within the ECS, where product proteins are periodically harvested. The secondary flow within the ECS transports these proteins to the downstream end of the bioreactor where they accumulate and increase the local osmotic pressure. Downstream polarization of medium proteins can

skew cell growth in this direction and cause a decrease in bioreactor productivity (Piret and Cooney 1990). However, this polarisation can concentrate the product proteins and harvesting from the downstream ECS port can facilitate product recovery and purification (Koska *et al.* 1997). One way of overcoming these gradients is the use of flat-bed hollow-fibre bioreactors. An example of this system is the Tecnomouse (Integra Biosciences, St Albans, UK). This bioreactor incorporates traditional hollow fibre technology with a direct oxygenation technique across a silicone membrane and thereby separates the aeration from the medium supply, and was designed for the production of antibodies from hybridomas. Typical antibody yields in the Tecnomouse are ~1.0-2.0 mg day^{-1} (Fassnacht *et al.* 1999). The Tecnomouse has also been used to culture transformed B- and T-cell lines and CHO cells to produce cytokines (Tzianbos and Smith 1995) and primary rat hepatocytes for potential use as a component of a bioartificial liver system (Bratch and Al Rubeai 2001). However, as with conventional HFBRs scale-up can only be achieved by increasing the number of units.

Hollow-fibre culture has mainly been used for the culture of suspension cells. This is due to the fact that anchorage-dependent cells do not readily attach to the cellulose acetate fibres. The cultivation of such cell lines can be achieved by coating the fibre walls with cell attachment material, such as poly-D-lysine (Hu and Peshwa 1991) or by using fibres made of polypropylene (Griffiths 1988). It may also be possible to use microcarrier beads as a cell substratum and load these into the ECS. Williams *et al.* (1997) successfully cultured CHO K1 cells on Cytodex™ 1 microcarriers in the ECS of a Setec Tricentric™ HFBR cartridge (Livermore, CA), and reached a final cell number of 1.4×10^9.

1.8 Cell encapsulation

The principle of cell encapsulation was originally developed by Nilsson and Mosbach (1980) and Lim and Sun (1980) and is used for the large-scale production of monoclonal antibodies (Rupp 1985). Although suspension cells can theoretically be immobilised in macroporous microcarriers, in practice the cell loading capacity is usually relatively low due to the non-adherent nature of the cells (Hu and Peshwa 1991). Thus, for suspension cells, such as hybridomas, it is more manageable to entrap cells rather than allow them to attach to solid particles. Suspended cells are mixed with alginate or agarose before droplets are formed to produce spherical particles. Alginates are naturally occurring polysaccharides extracted from seaweed, which have been commonly used in the biotechnology industry for numerous applications. In the case of sodium alginate beads, the particles are coated with polylysine, which provides a semi-permeable layer, allowing the transport of nutrients into the bead and toxic metabolites out of the bead. The sodium alginate is then solubilised with sodium citrate, releasing the cells into suspension. During the course of the culture, cells will proliferate within the capsule and produce antibodies, which are also retained within the structure, and very high concentrations of both can be achieved. A similar method is the use of calcium alginate only. This allows antibodies to diffuse out of the bead into the culture medium while cells are maintained within the capsule. Agarose beads can also be used where cells are encapsulated within a honeycomb matrix within the gel. However, these tend to have a wide size distribution and a lower mechanical strength compared to alginate gels (Griffiths 1988).

The main advantages of using alginate beads are:

- Encapsulated cells can be cultivated in conventional fermenters. Consequently, volumetric scale-up has greater potential than in hollow-fibre systems.
- The technology permits the cost effective production of monoclonal antibodies that are functional and highly purified.
- The process uses materials that are well established for use in the production of therapeutic agents.

Cell densities of $>10^8$ cells ml^{-1} have been reported using sodium alginate beads for microcapsule culture, which is significantly higher than the densities achieved in suspension culture (Rupp 1985). Calcium alginate beads can reach densities of ~7 x 10^6 cells ml^{-1} with a low viability of 56% through a 46 day run (Al Rubeai *et al.* 1990a).

One problem of major importance in all immobilised systems is the limited mass transfer rates (Al Rubeai and Spier 1989). Significant gradients of oxygen, nutrients and metabolites exist, resulting in uneven distribution of cell viability, metabolic activity and growth rate in the beads. Another disadvantage of using sodium alginate gels is that it is limited to batch operation with a culture time of 10-15 days. After this the capsules are lysed to allow the release of the product. The other methods of cell encapsulation do offer longer culture periods as the product is able to diffuse out of the bead and into the culture medium. However, it is difficult to maintain cell viability above 50%. These beads tend to be relatively large (0.5-1mm) which causes severe nutrient limitation in the centre, resulting in a necrotic region within the particle. It has been recommended that the bead diameter should be <0.5mm to maintain uniform distribution of metabolically active cells (Al Rubeai *et al.* 1990b).

1.9 Cell entrapment

Whilst cell immobilisation techniques such as hollow fibre bioreactors and gel encapsulation allow for high cell densities and increased product formation, the problems associated with their scale-up and the complexity of these systems has limited their suitability for industrial-scale processes (Wang *et al.* 1992; Yamaji *et al.* 1989). Originally, the alternative to these high density/low volume cultures was low density/high volume cultures using either suspension cells or solid microcarriers grown in stirred tank bioreactors. The disadvantage of these systems is that they are not cost effective in terms of capital expenses, time required for inoculum preparation and low cell densities and product titres, and cells were exposed to hydrodynamic forces and mechanical stresses during production, which limited cell specific product formation. The development of macroporous microcarriers has given manufacturers the option of having medium volume/high density cultures with high specific productivities during long-term cultivation. This immobilisation technique is passive in nature; cells can be seeded into a culture and will attach to the external surface of the carriers and grow in to the internal pores, thus being protected from mechanical damage caused by bead-bead collisions, bead-impeller collisions or bead-eddy interactions. Suspension cells can also be immobilised on macroporous carriers by forming aggregates within the porous structure. Hence, macroporous carriers can be used for both suspension and anchorage-dependent cell lines. The main advantages of macroporous microcarriers are summarised in Table 1.9.1.

Table 1.9.1

Advantages of macroporous microcarriers.

Unit cell density of 20- to 100-fold higher than solid microcarriers
Support both attached and suspension cells
in situ 100-250 seed expansion reducing scale-up steps
Suitable for stirred, fluidised or packed bed bioreactors
Short diffusion paths into a sphere
Good scale-up potential
Cells protected from hydrodynamic forces and mechanical stress
Capable of long-term continuous culture
Immobilisation in 3-D configuration, which is easily derivatised

Despite these numerous advantages some possible limitations exist. Following the growth of cells to tissue-like densities, the glucose consumption rate has been seen to decrease as a result of necrotic cores forming (Kennard and Piret 1994). Cells initially penetrate relatively deeply into the porous matrix. However, over time an outer shell of highly dense cells forms, limiting transfer of oxygen and nutrients to the centre of the particle (Al Rubeai *et al.* 1990b; Yamaji and Fukuda 1992). In addition to this, toxic metabolites such as lactate and ammonia are unable to diffuse out of the particle and CO_2 production will cause a drop in the pH. Consequently, this microenvironment induces cell death and a necrotic core develops, reducing the overall cell density and viability. This decreasing viability may therefore lead to unsatisfactory product yields. In a study by Preissmann *et al.* (1997) an assessment of limiting parameters for cells growing on macroporous microcarriers in a fluidised bed bioreactor was carried out. As no significant concentration gradients could be detected oxygen transfer to and into the carriers was examined. They found a significant transfer resistance within the laminar boundary film at the surface of the carrier and 40% air saturation in the bulk liquid could not provide efficient oxygenation during the exponential growth phase.

A further limitation in the use of macroporous microcarriers is in the production of viral vaccines. Viral production relies on the ability of virus particles to infect cells, replicate, and then release their progeny, usually by cell lysis. However, the accessibility of cells to viral infection may be a limiting factor when using macroporous microcarriers. Berry *et al.* (1999) compared the production of reovirus type-1 and type-3 from Vero cells cultivated on solid and macroporous microcarriers. Overall, the productivity in macroporous carriers was significantly lower than that from solid microcarriers and was likely to be due to the inaccessibility of entrapped

cells. They also concluded that the maximum cell yields were significantly lower when compared to values determined for solid microcarrier cultures.

There are currently a large number of macroporous microcarriers available which have been developed for stirred tank, packed-bed and fluidised bed bioreactors. The production of biologics within these bioreactor systems is described in Table 1.9.2.

Table 1.9.2

Characteristics of commercial macroporous microcarriers and their mode of use.

Microcarrier	Manufacturer	Matrix	Cell lines used
Cultisphere G	Percell Biolytica	Gelatine	CHO
Cytoline	Amersham Pharmacia Biotech	Polyethylene and silica	CHO Hybridoma
Cytopore	Amersham Pharmacia Biotech	Cross-linked cotton cellulose	CHO BHK
Fibra-cel	Bibby Sterilin	Polyester non-woven fibre and polypropylene	CHO Hybridoma BHK Vero FLYRD
Immobasil	Ashby Scientific	Silicone rubber	CHO HEK 293 BHK Hybridoma FLYRD
Microsphere	Verax Corp.	Collagen	CHO Hybridoma
Siran	Schott Glasswerke	Glass	CHO Hybridoma

1.9.1 Stirred Tank Bioreactors

Although many alternatives have been developed, the traditional stirred tank bioreactor has emerged as industry's technology of choice (Chu and Robinson 2001). The attractiveness of stirred tank bioreactors stems from their simplicity and ease of monitoring and controlling scale-up. Although they are commonly used for suspension cell culture, the use of macroporous microcarriers means that the same bioreactors can be used for immobilised anchorage-dependent cell lines. Suspension cells can also be entrapped within macroporous beads to increase the cell density. These cells are less dependent on attachment factors and Xiao *et al.* (1999) reported that the serum concentration could be reduced to as little as 0.1% to grow hybridomas, which are normally grown in 5-10% serum. This is an important consideration with respect to downstream processing and culture economics.

A typical characteristic of stirred tanks, and a major concern in animal cell technology, are the high mechanical forces that exist within the vessel. Not only are cells subjected to hydrodynamic forces from agitation when attached to solid microcarriers, but they are also vulnerable to high levels of energy released by bubble burst. Macroporous microcarriers may offer protection from these elements, provided that cells are able to migrate towards the centre of the particle. Many fibroblasts can move as fast as one centimetre a day on a smooth surface (Hu and Peshwa 1991) but the internal surface of these matrices is rarely flat or smooth and is quite tortuous for cells. However, once cells are protected from external forces the mixing of the stirred tank can be increased. This creates a completely homogeneous environment and allows for a greater oxygen and nutrient transfer rate to the immobilised cells (Xiao *et al.* 1999), resulting in high cell densities that range between 1 and 6 x 10^7 cells ml^{-1} microcarrier (Hu *et al.* 2000; Kennard and Piret 1994; Yamaji and Fukuda 1994), depending on

cell line and microcarrier used. Figure 1.9.1 shows CHO cells that have been grown to a high density on Immobasil FS microcarriers.

It has been observed that a decrease in specific growth rate occurs when immobilised suspension and anchorage dependent cells are grown in stirred tank perfusion cultures (Wagner *et al.* 1992; Yamaji and Fukuda 1994). Growth suppression has been proved to enhance antibody production in hybridoma cells. Therefore, immobilisation of cells may improve protein synthesis in stirred tank bioreactors. Certainly, the high cell densities can produce high yields of product. Stirred tanks have been used in the production of membrane anchored recombinant proteins, urokinase-type plasminogen activator, prourokinase, IgG and IgA monoclonal antibodies and vesicular stomatitis virus (Hu *et al.* 2000; Kennard and Piret 1995; Nikolai and Hu 1992; Schweikart *et al.* 1999; Xiao *et al.* 1999; Yamaji and Fukuda 1997).

The most appealing aspect of stirred tank bioreactors is the ease at which they can be scaled up in excess of 4000 litres, and this is undoubtedly why they have been so extensively used in industry. However, the inoculum density required is relatively high (when compared to microbial cultures). This problem is further increased when anchorage dependent cells are used if they have to be trypsinised at each step of the scale-up process. It has been shown that bead to bead cell transfer is possible with cytopore microcarriers (Xiao *et al.* 1999). A scale-up ratio of more than 20 was achieved meaning that only three scale-up steps are necessary for a 1000 l culture. This discovery has made the use of macroporous microcarriers a viable option for the commercial production of recombinant products from genetically engineered cells.

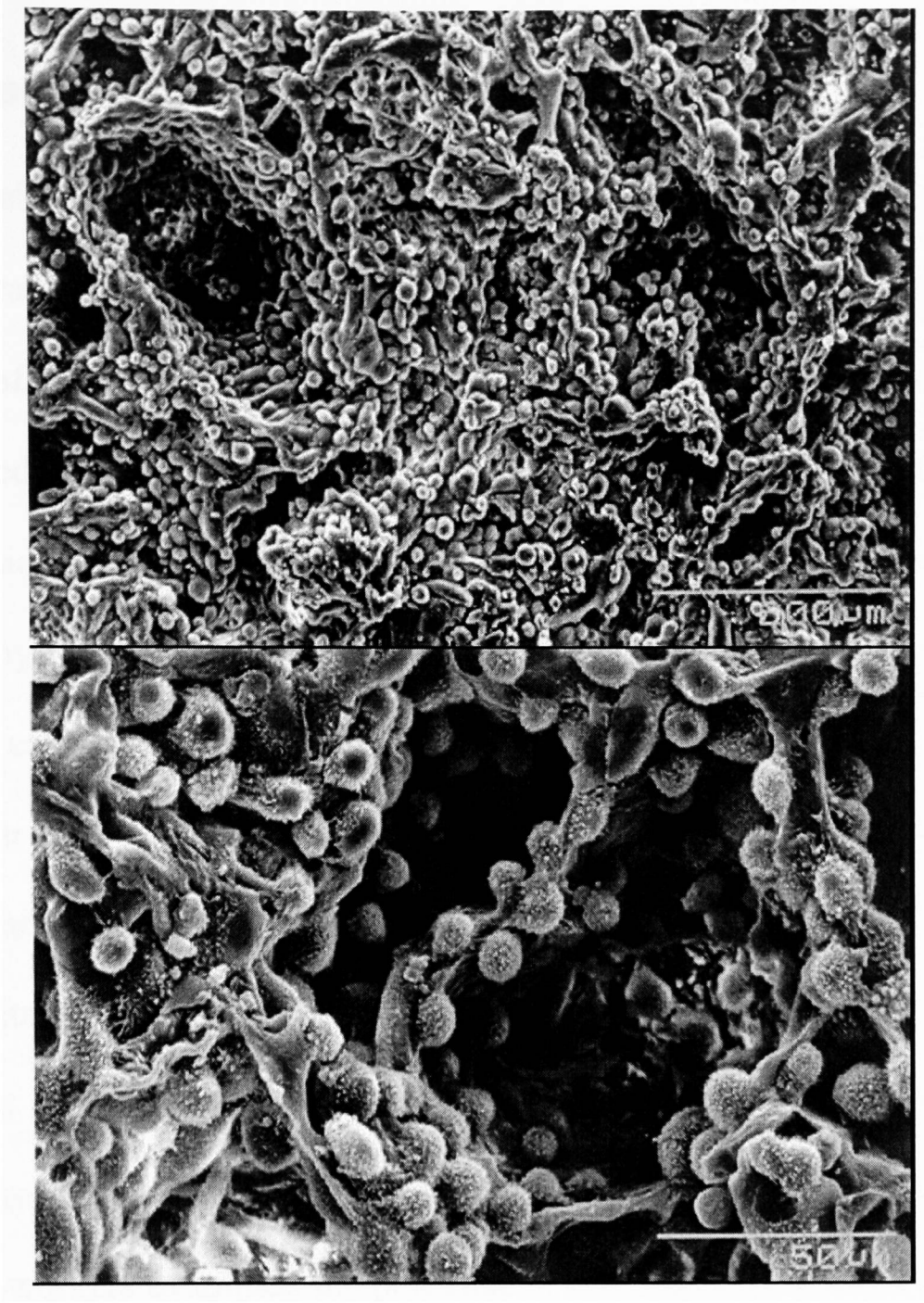


Figure 1.9.1

Scanning electron micrograph of CHO cells immobilised on Immobasil FS macroporous microcarriers.

1.9.2 Fluidised bed bioreactors

The most widely used culture system for porous microcarriers is the fluidised bed bioreactor (Kratje and Wagner 1992). The principle of their operation is that microcarriers of higher density than the culture medium are suspended by the upward flow of the medium, which is circulated through the bed. The height of the bed will increase as fluid flow increases. Medium circulation and fluid flow through the bed are achieved by using either a peristaltic pump or agitation. The advantage of this system is that carriers are separated from direct aeration and agitation, which avoids local high shear forces. In addition, fluidised bed bioreactors provide a very high mass transfer interface since both cells and fluid are moved (Preissmann *et al.* 1997) making it a suitable production system for larger scales.

Earlier studies into the potential of fluidised bed reactors for the production of biologics focussed on the use of porous glass beads. These studies conducted by Kratje and co-workers examined the potential of fluidised bed reactors as production systems using hybridomas suspension cells and anchorage dependent baby hamster kidney cells for the production of interleukin-2 (Kratje *et al.* 1994; Kratje and Wagner 1992). In these studies, cell densities of 3.8×10^8 cells ml^{-1} intrasphere volume were reached, whilst the IL-2 production rate was 0.75 mg l^{-1} day^{-1}. In spite of the cell density being over 18-fold higher, the productivity showed a 1.9 fold decrease when compared to a homogeneous stirred culture. The difference was assumed to be caused by cells changing their production characteristics during long-term cultivation. This phenomenon had previously been reported in hybridomas cells (Frame and Hu 1990). An alternative reason is that, unlike hybridoma cells, specific cell productivity is related to specific growth rate. . It has been shown by Leelavatcharamas *et al.* (1994) that there is a linear relationship between the production of interferon-γ and the

specific growth rate of CHO cells. Therefore, in long-term cultures where the cells remain in the stationary phase over an extended period, productivity will be low as the specific growth rate is essentially zero.

Another substrate for the immobilisation of cells grown in fluidised bed bioreactors is polyethylene and silica microcarriers, such as Cytoline (Amersham Pharmacia Biotech, Sweden). These have been successfully used in the production of recombinant human proteins, especially human interferon-γ, and erythropoietin (Goldman *et al.* 1998; Wang *et al.* 2002). These microcarriers have been specifically designed for use in the Cytopilot fluidised bed bioreactor and have proved to offer great potential for the production of biologics. Kong *et al.* (1999) compared CHO cell cultures in this system with solid microcarrier culture in a stirred tank bioreactor and reported that the product output rate was approximately three times higher in the fluidised bed, with the specific production rate being 5.5 times higher. The volumetric productivity of the stirred tank reached 5.6 mg l^{-1} day^{-1} during the growth period. However, after the maximum cell density was reached the volumetric productivity dropped and was maintained around 1.65 mg l^{-1} day^{-1}. In contrast, the Fluidised bed bioreactor maintained a consistent volumetric productivity of 22.8 mg l^{-1} day^{-1}. It was suggested that the isolated aeration and agitation in the fluidised bed bioreactor reduced the amount of hydrodynamic and mechanical stress imposed on the cells. Thus, more cellular energy was utilised in product formation, resulting in the higher and more consistent productivity. The total product yield was reported as 34.2 mg day^{-1} and 13.2 mg day^{-1} for the 2 litre fluidised bed bioreactor and the 10 litre stirred tank bioreactor, respectively.

When comparing the number of cells in each square meter of microcarrier surface area the Fluidised Bed Reactor was five times lower than the stirred tank reactor. This

shows that there is further potential to increase the cell number and improve the productivity of the system. In order to unlock this potential it is necessary to ascertain the limiting factor for growth and how to overcome this limitation. Investigations carried out by Preissmann *et al.* (1997) determined that the transport of oxygen to cells within the macroporous matrix cultivated in a fluidised bed bioreactor was severely limited, leading to poor process performance. Although no limitations in nutrient concentration were detected, the beads were not believed to be an ideal substrate to grow cells to tissue like densities.

Efficient glycosylation is an important factor in the development of a bioprocess production method. The existence of gradients that cause decreases in dissolved oxygen, nutrients or pH may have implications for product quality, as well as cell densities. In a recent study, the relative proportion of glycosylation site occupancy for interferon-γ was not seen to change over time during a 500 hour culture. Hence, it was concluded that long-term perfusion cultures of CHO cells in a fluidised bed bioreactor could produce IFN-γ with a consistent and highly comparable degree of glycosylation (Goldman *et al.* 1998). Further studies, carried out by Wang *et al.* (2002), have also examined the productivity and quality of erythropoietin (EPO) from a recombinant CHO cell line, cultured in a Cytopilot fluidised bed bioreactor. EPO is particularly vulnerable to a decline in sialylation due to the extensive level of glycosylation and heterogeneity. It was therefore important to show that the glycosylation profile of EPO in the Cytopilot did not differ from other culture systems. Results have shown that no difference was observed in electrophoretic product profiles between fluidised bed, stirred tank and stationary cultures. In addition, the significantly higher productivity rate in the fluidised bed bioreactor, when compared to stirred tank

reactors, makes it an attractive option for the large-scale production of recombinant proteins.

A potential problem that has been observed during the long-term cultivation of cells in medium containing 10% foetal calf serum is the clumping of carriers. This clumping causes a reduction in bed height, which requires increased agitation and fluid flow to maintain sufficient fluidisation. The reason for carriers clumping is not fully understood but it may be due to cell bridging. This is an incident usually associated with solid microcarriers, where cells become simultaneously attached to two beads, thus creating a bridge. A possible means of overcoming this problem is to reduce the concentration of serum used in the process. This could be done step wise during the course of the experiment if serum is necessary for cell attachment and growth in the initial stages. The clumping of carriers has not been observed to have an adverse effect on productivity. It does, however, limit the duration of the process as excessive clumping has an adverse effect on reactor performance.

1.9.3 Packed bed bioreactors

Packed bed bioreactors have been used for the cultivation and production of a wide range of cell lines and biologics. Some recent examples include monoclonal antibody, anti-leukaemic factor from stromal cells, recombinant proteins (e.g. recombinant Ca^{2+} binding receptor and HIV-1 gp120), and retrovirus vectors for use in gene therapy (Hu *et al.* 2000; Kadouri and Zipori 1989; Kang *et al.* 2000; Kaufman *et al.* 2000; Merten *et al.* 2001; Wang *et al.* 1992). Since the initial conception of packed-bed bioreactors for the production of biologics in the 1970's, they have grown in acceptance and their potential as a viable system for commercial manufacture of animal cell products. Stirred tank and fluidised bed bioreactor systems have been scaled up in excess of 1000 litres and have been widely used in industrial processes

(Cong *et al.* 2001). However, at high cell densities, cells detach from the carriers and along with cell debris cause complications in product purification during downstream processing. Packed bed bioreactors have the advantage that they are capable of generating high cell densities with a low concentration of free cells in suspension. This is possible due to the low shear forces present in the system. Cells are immobilised within porous carriers that may be porous ceramic beads (Park and Stephanopoulos 1993), porous glass beads (Chiou *et al.* 1991), or polyester disks (Hu *et al.* 2000; Kang *et al.* 2000; Wang *et al.* 1992), which are packed and retained in a cylindrical vessel through which culture medium is re-circulated. As mentioned earlier, a characteristic problem in intensive production systems is the reduced transport rate of limiting nutrients, such as oxygen to immobilised cells, which restricts their final density. Packed bed technology has overcome this problem by the use of intraparticle convective flow. Through the development of a simple hydrodynamic model based on the Blake-Kozeny equation, Park and Stephanopoulos (1993) significantly enhanced the transport of oxygen in a packed bed, allowing the maintenance of cell viability and productivity whilst sustaining a low shear environment. In their evaluation of the model they achieved cell densities of 5.1 x 10^8cells ml^{-1}. They also calculated the specific insulin productivity to be 0.88 x 10^{-5} μU $cell^{-1}$ $hour^{-1}$. The model developed allows packed bed bioreactors to be scaled-up several-fold before oxygen becomes limiting. Any oxygen limitations that do occur primarily exist close to the bioreactor exit. Another recent development to overcome this problem has been to provide oxygenation to the bed through silicone tubing. This simple but effective method has been used for the successful culture of hepatocytes and the production of retrovirus particles from the human packaging cell lines, FLYRD18 and TEFLYRD (McTaggart and Al Rubeai 2000).

An alternative system is the Celligen® packed-bed bioreactor, developed by New Brunswick Scientific Co. (New Jersey, USA). Conventional packed beds require a conditioning vessel to control culture parameters such as temperature, dissolved oxygen and pH. The culture medium is circulated through the packed bed and the product may be concentrated and continuously or periodically harvested from the conditioning vessel for prolonged periods. An important parameter in the optimisation of these arrangements is the circulation rate; if the rate is too low then gradients in pH, nutrients and metabolites will appear along the bed affecting cell viability, productivity and product quality, as can be seen in hollow fibre bioreactors (Thelwell and Brindle 1999). If the circulation rate is too high it may have an adverse affect on cell attachment or product formation. This problem is eliminated in the Celligen® packed bed bioreactor by the fact that the bed is contained in a basket within a stirred tank bioreactor. Mixing in the bioreactor is achieved with either a cell-lift impeller, which allows bubble-free medium to flow through the packed bed (Wang *et al.* 1992) or a double screen concentric cylindrical cage impeller. Shi *et al.* (1992) have shown that the latter impeller is able to increase convective mass transfer, cell concentration and MAb product concentration. The significant improvements in oxygen transfer rate, cell density and product concentration were attributed to the increased surface area allowing for convective oxygen transfer and protection of cells from hydrodynamic stresses. Both methods create a homogeneous environment for cells to grow and product can be directly removed from the culture vessel, which allows for easier downstream processing and product purification.

One drawback of large-scale packed bed bioreactors using anchorage-dependant cells is the generation of seed stock. Cultures are commonly inoculated at a density of 2 x 10^5 cells ml^{-1} of bed volume, which could be as high as 20 litres in a 100 litre vessel at

commercial scale. Scale-up is typically done from monolayer cultures using either T-flasks or roller bottles due to difficulties in harvesting cells from macroporous microcarriers and low cell densities achieved from solid microcarrier culture. Thus, while the production culture may be simple and cost effective the scale-up steps may be less economical and susceptible to contamination. In a recent study, a method has been devised whereby cells are scaled-up on Cytopore macroporous microcarriers and the carriers are directly inoculated into the packed bed bioreactor, alleviating the need to detach cells from the carriers (Cong *et al.* 2001). Using this method, it was possible to achieve a cell density of 2×10^7 cells ml^{-1} bed volume and thrombopoietin production was 1.3-1.8 mg l^{-1} day^{-1}, compared to 0.76-1.1 mg l^{-1} day^{-1} for macroporous microcarrier perfusion culture. Cultures were maintained in excess of 30 days.

A further disadvantage of packed bed bioreactors is the inaccessibility of carriers, making it difficult to monitor viable cell number. Cellular metabolism can be followed by online measurements of pH and dissolved oxygen and offline determinations of glucose, lactate, ammonia and glutamine. The substrate uptake rates and production rates are conventionally used as cell growth markers (Rodrigues *et al.* 1999). As cell concentration generally has a linear correlation with glucose uptake rate at high cell densities it is possible to calculate the former from the latter (Portner *et al.* 1994). However, the yield coefficient is not a true constant and will vary for each cell line and for each bioreactor system. Therefore, it should be determined before being used to estimate cell number. Alternatively, cell number can be determined through measuring the oxygen uptake rate (OUR). By placing oxygen probes upstream and downstream of the packed bed and measuring the percentages of dissolved oxygen, it is possible to determine the OUR, which can be directly

correlated with cell number. The density of cells within an immobilised culture is an important parameter for many purposes including calculation of the specific productivity of a system.

1.10 Summary

Gene therapy has revolutionised the practice of medicine to treat patients with inherited or acquired diseases (Mountain 2000). The delivery of functional genes to host cells means it is now possible to correct dysfunctional genes or invoke an immune response. A number of gene delivery vehicles exist, either viral or non-viral. Non-viral vectors are considered safer than viral vectors but are susceptible to extracellular degradation and have very low transduction efficiencies. In contrast, viruses have evolved into extremely efficient vectors for gene transfer. Current research is investigating the use of retro-, adeno-, adeno-associated, herpes and alpha viruses as gene therapy vectors. Of these, retroviruses comprise >60% of the current clinical trials. Retroviruses are appealing because they are able to permanently insert their viral sequence into the cell genome without any adverse effect on cellular growth or function.

Retroviral vectors are generated by deleting the helper gene sequences from the viral genome and replacing them with a therapeutic gene and a selectable marker. The viral proteins, gag, pol and env are produced in packaging cells, which contain plasmids expressing their genes. Vector particles assemble within the producer cells and are continuously released into the supernatant through budding. Packaging cells are unable to produce viral particles unless they have been infected with the vector as they do not contain the Ψ packaging sequence or LTRs. Research into the molecular biology of retrovirus systems has largely overcome the problems associated with low viral titres, inactivation by complement in human serum and the generation of RCR

through recombination. However a number of biochemical engineering challenges remain and there is an urgent need to transpose production of clinical grade vectors into bioreactor systems.

Traditional stirred tank reactors using suspension cells are still the preferred choice of biopharmaceutical production in the biotech industry. However, due to the relatively low cell densities, immobilised cell cultures are growing in interest for the production of high value biologics such as gene therapy products. Immobilised cultures offer many advantages such as near tissue cell densities leading to higher productivity, increased specific production rates, separation of products from cells, improving the efficiency of the downstream processing, serum concentration can be reduced making the process more economical and immobilisation techniques can be used for both suspension and anchorage-dependent cells.

A number of systems can be used in the production of biologics from immobilised cell cultures. The advantages and disadvantages of these systems are summarised in Table 1.10.1.

Table 1.10.1

Advantages and disadvantages of cell immobilisation systems for the production of biopharmaceutical products from animal cells.

Production System	Advantages	Disadvantages
Hollow Fibre Bioreactor	$>10^8$ cell ml^{-1} 200-300 fold increase in product Reduced serum concentration	Poor cell viability Diffusion gradients exist Limited scale-up Difficult to culture anchorage-dependent cells
Alginate Beads	Uses conventional fermenters Cost effective Uses materials well established in biotech industry	Limited mass transfer rates Sodium alginate beads limited to batch culture Cell viability <50%
Stirred Tank Bioreactor	Simple to operate Cells protected from mechanical stresses Growth suppression leads to enhanced productivity Scale-up >1000 litres	Lower cell concentration compared to other systems Unable to support high microcarrier density
Fluidised Bed Bioreactor	Carriers separated from agitation and aeration Consistent volumetric productivity Consistent product quality Good scale-up potential	Specific productivity reduced in some cell lines Oxygen limitations may occur Carriers clump at high serum concentrations
Packed bed Bioreactor	Generate and retain high cell densities Intraparticle flow restricts O_2 limitations	Difficult to directly measure cell density Difficult to generate anchorage-dependent cells for inoculation

1.11 Aims and objectives

To enable the optimisation of a bioreaction process in animal cell culture, the process has to be designed so as to fully exploit the biological potential of the cell. This is done through maximisation of viable cell number and improvement of the medium formulation. Manipulation of the physical environment, by altering the mode of bioreaction and/or the bioreactor system, is the basic method for improving cell number (Leelavatcharamas *et al.* 1994). In this study, retrovirus production was predominantly examined in semi-continuous and perfusion cultures. A selection of microcarriers was assessed with regard to their potential as a cell substrate for immobilised culture and a number of bioreactor systems were evaluated. These included stirred tank bioreactors, packed bed bioreactors and fluidised bed bioreactors. Finally, the composition of culture medium, and the initial concentration of serum was analysed, and growth and production kinetics were determined by mathematical modelling.

The overall objective of this thesis was to propose an economical, efficient and scalable process that could be implemented by Oxford BioMedica for the production of their MetXia® gene therapy product.

CHAPTER 2: GENERAL MATERIALS AND METHODS

This chapter describes the materials and methods that were used throughout this research work. Details of specific protocols are given, where appropriate, in individual chapters.

2.1 Cell Lines

2.1.1 TEFLYRD/83

The TEFLYRD packaging cell line was constructed from the TE671 cell line (Takeuchi *et al.* 1994). The transfected cell line contained two plasmids; the first expresses the Moloney murine leukaemia virus (MoMLV) *gag-pol* gene and the second expresses the *env* gene originating from the cat endogenous virus, RD114.

TEFLYRD cells were used by Oxford BioMedica to construct the TEFLYRD/83 cell line. In brief, the cells were transfected with a third plasmid containing the G418/neomycin resistance gene (as a selectable marker), the *LacZ* gene and the MetXia-P450 gene. The MetXia-P450 gene encodes the MetXia® therapeutic protein which is a potential treatment for a range of solid tumours. In recent phase I/II clinical trials it was shown that MetXia® was capable of reducing the size of tumours and that it may also elicit an anti-tumour immune response, destroying other tumours in the same patient (Kan *et al.* 2001).

2.1.2 HT1080

The human fibrosarcoma HT1080 cell line (ATCC CCL-121) was kindly donated by Oxford BioMedica (Oxford, UK). These was used as the target cells to measure

retrovirus particles with the LacZ titration assay (see 2.5 Measurement of Lac Z Containing Retroviral Vectors).

2.2 Recovery and Maintenance of Cells

2.2.1 Cell Freezing

A cell bank was created and stored in liquid nitrogen. The cells were passaged as described in 2.2.3. After the cells had been trypsinised they were centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 70% Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies, UK), 20% Foetal Calf Serum (FCS) (PAA Laboratories, UK), and 10% Dimethyl sulphoxide (DMSO) (Sigma, UK). The cell suspension was alloquoted into 1 ml cryotubes. These were placed in a freezing container, containing iso-propanol, and stored in a freezer at -80°C. The iso-propanol allowed the temperature to drop by 1°C every minute, preventing the formation of crystals that would kill the cells. After a minimum of 90 minutes the cells were transferred from the -80°C freezer to liquid nitrogen.

2.2.2 Cell Revival

The cells were removed from liquid nitrogen and defrosted at room temperature. They were added to 10 ml of DMEM + 10% FCS in a sterile universal tube and centrifuged for 5 minutes at 1000rpm. The supernatant was discarded and the pellet resuspended in 10 ml of DMEM + 10% FCS. Cells were counted by removing 100µl of suspension and adding 0.5% trypan blue at a ratio of 1:1. The cells were counted under a microscope using a heamocytometer counting chamber. They were then inoculated into T-flasks at a concentration of 2×10^4 cells cm^{-2}. For 25 cm^2 and 75 cm^2 T-flasks

the total volume of medium was made up to 10 ml and 20 ml, respectively. After 24 hours the medium was removed and replaced with fresh medium.

2.2.3 Cell Maintenance

Cells were routinely maintained in 75 cm^2 tissue culture flasks (T-flasks) with 20 ml of DMEM containing 4.5 g l^{-1} of glucose, supplemented with 10% (v/v) FCS, and 100 IU ml^{-1} penicillin streptomycin (Gibco Life Technologies, UK) to prevent bacterial contamination. The cells were passaged twice weekly.

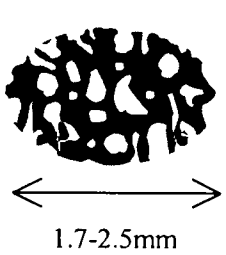
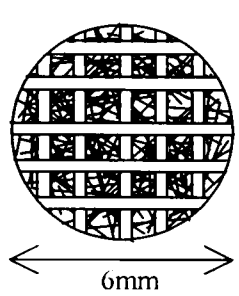
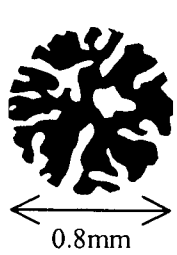
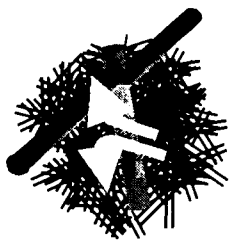
Cells were passaged using standard trypsinisation methods. In brief, medium was removed from T-flasks and the cells were washed with sterile phosphate buffered saline (PBS). Cells were detached by treating with trypsin-EDTA (Gibco Life Technologies, UK) for approximately 5 minutes. Once the cells had detached from the surface of the flask DMEM + 10% FCS was added to neutralise the trypsin. T-flasks were seeded with the cell solution at an appropriate density and the total amount of medium was made up to a suitable volume. The flasks were incubated at 37°C in a 5% CO_2 atmosphere.

2.3 Preparation of Microcarriers

Cytoline 1 and Cytoline 2 microcarriers were supplied by Amersham Pharmacia Biotech (Upsala, Sweden), Fibra-Cel™ microcarriers were obtained from Bibby Sterilin (UK) and Immobasil-FS carriers were provided by Ashby Scientific Ltd. (UK). The specifications of these microcarriers are described in Table 2.1.

Table 2.1

Technical specifications of microcarriers.

	Cytoline 1	Cytoline 2	Fibra-Cel	Immobasil-FS
Matrix	Polyethylene and silica	Polyethylene and silica	Polyester non-woven fibre & polypropylene (50/50)	Silicone rubber
Average particle size (mm)	Thickness: 0.5-1 Length: 1.7-2.5	As Cytoline 1	Diameter: 6.0	Diameter: 0.8 Depth: 0.25
Schematic representation				
Anticipated fluid flow	 *	 †	 #	
Approximate surface area ($m^2 g^{-1}$)	> 0.3	> 0.1	0.12	
Pore diameter (μm)	10-400	10-400		50-150

* Fluid may enter the larger pores (>200μm) where may be cells exposed to shear forces. Fluid is not expected to enter smaller pores, and a microenvironment is expected to form.

† Fluid is not expected to penetrate the densely woven fibres. However, forced convection in a packed bed assists the transport to and from the disc.

Fluid does not enter pores. A microenvironment forms and transport of virus particles into the medium may be hindered.

2.3.1 Cytoline 1 & 2

The preparation of Cytoline 1 and Cytoline 2 microcarriers was identical. To the desired amount of microcarriers twice the volume of double distilled water was added and the mixture was autoclaved for 10 minutes at 121°C (1 bar). The distilled water was removed from the mixture and replaced with twice the amount of fresh double distilled water. The mixture was stirred for approximately 10 minutes. The double distilled water was removed and the process repeated three times. After the third wash and equal volume of 0.1M NaOH was added to the microcarriers. The mixture was incubated overnight at room temperature. The mixture was washed 3-4 times with double distilled water to remove all the alkali. The microcarriers were transferred to the culture vessel and autoclaved in double distilled water for 30 minutes at 121°C (1 bar). The double distilled water was removed aseptically, and replaced with culture medium. The mixture was incubated overnight under culture conditions to allow the microcarriers to equilibrate.

2.3.2 Fibra-Cel™

The desired amount of discs was placed in the culture vessel and PBS was added. The vessel was sealed and autoclaved at 121°C, 1 bar, for 30 minutes. The PBS was aspirated and growth medium was added to the vessel to cover the carriers. The carriers were incubated under culture conditions for a minimum of 24 hours to allow equilibration and to check for sterility. Immediately prior to inoculation the medium was removed.

2.3.3 Immobasil-FS

Immobasil-FS carriers were supplied ready hydrated by the manufacturer. The required volume of beads was added to the culture vessel with an equal volume of double distilled water. The carriers were autoclaved at 121°C, 1 bar, for 30 minutes. Following sterilisation, the DD water was removed and the carriers were equilibrated for at least 24 hours in growth medium under culture conditions, and to verify sterility. Before inoculation the equilibration medium was removed and discarded.

2.4 Monitoring of Cell Growth and Cell Adhesion

2.4.1 Counting of Attached Cells in Tissue Culture Flasks

Cells were detached from the flask surface as described in 2.2.3 Cell Maintenance. A 100µl cell suspension was removed and stained with an equal volume of trypan blue solution (0.5% w/v at 1:1 dilution). Viability was determined using the dye exclusion test. Viable cells (unstained) and non-viable cells (stained) were counted under a microscope using a heamocytometer chamber.

2.4.2 Measurement of metabolic parameters

The glucose consumption and lactate production were determined by measuring the concentration in fresh and exhausted medium. The concentration of glucose in the culture supernatant was determined using a GLUCOTREND® (Roche, France) test strip kit as per the manufacturer's instructions. Lactate was measured using the Biolyser Rapid Analysis System (Eastman Kodak Co., USA). A 10µl sample was added to a slide containing the appropriate reagents and the relative concentration was determined as per the manufacturer's instructions.

2.4.3 Estimation of cell number using an MTT Assay

The MTT assay (Mosmann 1983) is a sensitive, quantitative and reliable colorimetric method of estimating viable cell number. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethyliazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product, which is insoluble in water. The amount of formazan production is proportional to the number of cells in a variety of cell lines (Al Rubeai *et al.* 1990; Mosmann 1983). As the assay does not require the cells to be in suspension it can be used to estimate cell number in microcarrier cultures where trypsinisation is not appropriate.

A stock solution of 5mg ml^{-1} of MTT (Sigma, UK) in PBS was prepared and filtered through a 0.22μm filter to sterilise and remove the small amount of insoluble residue. This stock solution was stored at 4°C for a maximum of 1 month. A known number of microcarriers, typically 20-50, were suspended in 1ml of culture medium and 100 μl of MTT was added. The samples were incubated at 37°C for 3 hours to allow the MTT to diffuse through the matrices and react with the cells. The supernatant was removed and replaced with 1 ml of acidic iso-propanol. The samples were shaken at 1000rpm for 20 minutes using a Vibramax shaker to release the formazan products from the microcarriers. 100 μl of the formazan solution was pipetted into individual wells of a 96 well plate and the absorbance was measured at 540nm using an Elisa plate reader (SLT Spectra, UK).

2.4.4 Observation of Cell Adhesion

Fluorescence Microscopy

Approximately 1 ml of microcarriers were placed into a 25 ml universal tube and the supernatant was removed. The carriers were washed once with PBS. The PBS was removed and replaced with acridine-orange solution (4 $\mu g\ ml^{-1}$). The DNA stained cells were observed under a fluorescent microscope.

2.5 Measurement of Lac Z Containing Retroviral Vectors

Active virus titres were assessed by either the LacZ titration assay or by real-time polymerase chain reaction (RT-PCR).

2.5.1 LacZ Titration Assay

The number of MetXia-P450 vector particles in a given preparation can be determined by their ability to transfer the *lacZ* gene to indicator cells, in a simple titration assay. Successful gene transfer and the subsequent expression of β-galactosidase are assessed by X-Gal staining. At the end of the assay the number of particles in the preparation is expressed as *lacZ* transferring units per ml (LTU ml^{-1}).

Day 1: Plating HT1080 Indicator Cells

First, the number of 12 well plates required was calculated. Typically, 2-3 dilutions of each sample were plated. Therefore:

$$\frac{no.\ of\ samples \times no.\ of\ dilutions \times no.\ of\ replicates}{no.\ of\ wells} = no.\ of\ plates$$

Each well of the 12 well tissue culture plates was seeded with 10^5 target cells. In order to ensure inter assay consistency, fresh HT1080 cells were revived from a working bank laid down specifically for this purpose. The cells were used within five passages of resuscitation. The plates were incubated at 37°C and 5% CO_2 overnight.

Day 2: 10x Serial Dilution of Virus

A 10-fold dilution series of the test article was made in DMEM supplemented with 8μg ml^{-1} of polybrene (Sigma, UK) in a 24 well tissue culture plate. 1080μl of the polybrene-supplemented medium was placed into each well of the plate. 120μl of vector preparation was added to well A1 and mixed thoroughly. 120μl was then transferred from well A1 to well B1; this process continued down the next 4 wells to create the dilution series. The diluted vector preparations were then used to transduce the indicator cells that were prepared on day 1. The culture medium was removed from the growing target cells and they were overlaid with 0.5ml of vector from the appropriate dilution wells. This process was carried out in duplicate. The plates were incubated at 37°C and 5% CO_2 for two hours before a further 1ml of fresh medium was added to each well. Incubation was continued for another 48 hours.

Day 4: X-Gal Staining

The medium was removed from each well and replaced with 0.5 ml of 4% neutral buffered formalin solution (BDH Chemicals, UK). This was incubated at room temperature for 5 minutes. The fixing solution was carefully removed and the cells washed once with 1ml of PBS. 0.5ml of X-Gal stain was added to each well and incubated at 37°C for 24 hours.

The X-Gal stain was prepared as required:

2ml potassium ferricyanide (Sigma, UK)

2ml potassium ferrocyanide (Sigma, UK)

100μl 1M magnesium chloride (Sigma, UK)

1ml 2% X-Gal solution (Alexis, UK)

It was made up to 50ml with sterile PBS.

The solutions were added in order to prevent a precipitation from occurring.

Day 5: Cell Counting

On the fifth day the transduced cells were counted under a microscope. A single transduction event was represented by a small cluster of blue cells, which were the progeny of a transduced cell that divided during the course of the assay. The value was obtained by counting the cell clusters in wells where the number of clusters was between 30 and 300.

The final titre was calculated by multiplying the mean number of clusters from duplicate wells by the dilution factor and then multiplying by 2 to convert to a ml, thus giving the number of *lacZ* transferring units per ml (LTU ml^{-1}).

2.5.2 Real Time PCR

Real time PCR is a reliable and rapid method for the enumeration of infectious virus particles. The reaction exploits the 5′ nuclease activity of AmplTaq Gold® DNA polymerase to cleave a Taqman probe during PCR. The Taqman probe contains a reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe. During the reaction, cleavage of the probe separates the reporter and the quencher dyes, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, the probe specifically anneals between the forward and reverse primer sites if the target of interest is present. Cleavage of the probe between the reporter and quencher dyes only occurs when the probes hybridises to the target. The probe fragments are then displaced from the target, and polymerisation of the strand continues. The 3′ end of the probe is

blocked to prevent extension of the probe during PCR. This process occurs during every cycle and does not interfere with product accumulation.

In brief, supernatant containing retrovirus was boiled to rupture the viral envelope and release the RNA genome. Reverse transcriptase was added to the solution to generate DNA. The amount of DNA was measured by real time PCR using the Taqman probe. The results obtained from RT-PCR are expressed relative to some basis sample, such as a calibrator. Therefore, the virus titre in all experimental samples was determined from a standard curve, which was prepared from a known quantity of virus particles.

Chapter 3: Fundamental characterisation of TEFLYRD cells and OB83 retroviral vector particles

3.1 Introduction

Preliminary experiments were conducted to determine the fundamental characteristics of the TEFLYRD/83 cell line and the vector particles produced before any bioreaction or medium modifications were performed. These studies included construction of an MTT calibration curve, characterisation of cells and vector production in batch culture and long-term semi-continuous culture at 37°C and 32°C, and the attachment and growth of cells on various microcarriers under static and stirred conditions.

The results from these experiments provided a basic understanding of the packaging cells and the vectors they produced, which assisted in the planning of future experiments.

3.1.1 Microcarrier culture

Animal cells are widely used for the production of biologics including viral vaccines, recombinant proteins, monoclonal antibodies, hormones, interferons, and gene therapy vectors (Berry *et al.* 1999; Croughan *et al.* 1987; Forestell *et al.* 1992; Hu and Peshwa 1991; Nikolai and Hu 1992; Venkat *et al.* 1996; Yamaji *et al.* 1989; Zecchini and Smith 1999). A number of cell lines currently in use are anchorage dependent. One of the earliest technologies for the culture of these cells and the production of biologics was roller bottles (Schevitz *et al.* 1990). Whilst this method is fitting for developmental work it is not an attractive option for large-scale manufacture (Zecchini and Smith 1999). This is because scale-up can only be achieved by increasing the number of bottles, which is labour intensive and susceptible to contamination (Butler 1987). Modern facilities have overcome these problems by the

use of expensive robots; however, it is still not possible to control environmental conditions such as pH or dissolved oxygen levels (Hu and Peshwa 1991). Microcarriers were first described by van Wezel (1967) and offer a scalable alternative to roller bottles for the culture of anchorage dependent cells. They present a solid surface for cells to adhere to and can be easily suspended in liquid medium within a stirred tank reactor (Christi 1993).

The first microcarriers to be developed were solid spheres made up of cross-linked dextran. Others carriers have subsequently been developed using alternative materials such as polystyrene, cellulose, gelatine and glass (see review by Hu and Peshwa 1991). The advantages of using microcarriers are that they offer growth-surface-to-reactor-volume ratios of over $100cm^{-1}$, they allow for simple medium and cell separation and perfusion, they can support high cell densities ($>10^6$ cells ml^{-1}), and can be easily scaled-up (Croughan *et al.* 1987). Solid microcarriers do have some disadvantages, though. The spheres have a low surface-area to volume ratio, limiting the number of cells per bead and are vulnerable to hydrodynamic forces in the culture (Ng *et al.* 1996). The mechanisms of cell damage in microcarrier culture are primarily caused by collisions between beads and reactor parts, principally the impeller, collisions with other microcarriers and interactions with turbulent fluid eddies of the same size or smaller than the microcarrier diameter (Cherry and Papoutsakis 1988). In order to overcome these problems, macroporous microcarriers were developed.

The concept of macroporous microcarriers was pioneered by the Verax Corporation and there are currently large numbers of carriers available for use in stirred tank, fluidised bed and fixed bed reactors (Griffiths 1992; Looby and Griffiths 1990). These beads have relatively large internal pores and are typically made of gelatine or collagen (Nikolai and Hu 1992). After inoculation, cells attach to the surface of the carriers and then migrate inwards, eventually occupying the interior of the bead.

Unlike solid carriers, the cells immobilised within the structure are not in direct contact with the fluid flow and are not thought to be prone to any mechanical damage caused by agitation, bead interactions, or turbulence (Hu and Peshwa 1991; Nikolai and Hu 1992). Due to the large internal matrix, macroporous microcarriers provide a greater surface area per carrier for cell expansion than solid carriers. The cell concentration is, therefore, potentially much higher than with conventional microcarriers (if a comparable amount of beads are used) or suspension cultures (Hu and Peshwa 1991; Nikolai and Hu 1992).

Virus productivity is dependent on several culture parameters, including the use of a suitable host cell and the type of microcarrier used (Berry *et al.* 1999). Varani *et al.* (1998) have shown that productivity is dependent upon the virus, host cell line and the microcarrier type. Therefore, the ideal microcarrier should i) support rapid cell attachment; ii) support rapid high-density cell growth; iii) not interfere with the secretion of products; and iv) allow cells to be easily detached if required (Varani *et al.* 1998). In this study, four microcarriers have been evaluated with regard to these criteria. The microcarriers used were Cytoline 1 and 2 (Amersham Pharmacia, Sweden), Immobasill FS (Ashby Scientific, UK) and Fibra-Cel™ (Bibby-Sterilin, UK).

3.2 Materials and methods

3.2.1 MTT calibration curve

In order to determine the cell number from absorbance a calibration curve was constructed using TEFLYRD/83 cells. In brief, duplicate 1ml suspensions of known cell number, between 1 x10^6 cells ml^{-1} and 1.25 x 10^5 cells ml^{-1}, were added to eppendorf tubes and 100µl of MTT was added. These were incubated at 37°C for 2 hours. The cell suspension was then centrifuged at 10000 rpm for 2 minutes in an Eppendorf 5415C centrifuge. The supernatant was removed and the cells were resuspended in 1 ml of acidic iso-propanol. The tubes were shaken for 20 minutes at 1000 rpm in a Vibramax shaker. 100 µl of the formazan solution was pipetted into individual wells of a 96 well plate and the absorbance was measured at 540nm using an SLT Spectra Elisa plate reader.

3.2.2 Batch culture of TEFLYRD/83

The typical behaviour of TEFLYRD/83 cells, with regard to cell growth and vector production, was assessed over a 10-day period. Monolayer cultures were established in several T-25 culture flasks using 10 ml DMEM supplemented with 10% FCS (as described in chapter 2) and were inoculated with ~6 x 10^5 cells. Flasks were incubated at 37°C with 5% CO_2. Cells were trypsinised from duplicate flasks at regular intervals and counted under a microscope with a heamocytometer. Viability was determined using the trypan blue exclusion test. The supernatant was collected and the concentration of glucose and lactate was measured. A 1ml sample was stored in liquid nitrogen for virus analysis using the LacZ titration assay at a later point. The number of cells per flask, glucose consumption, lactate production and virus production were assumed to be constant under identical culture conditions.

Data Analysis

Cell growth is a first order autocatalytic process i.e. $r_x = \mu x$. Taking the time period of the early exponential growth phase, specific growth rate, μ, can be calculated given that:

$$\frac{dx}{dt} = r_x = \mu x$$

The specific growth rate changes in a hyperbolic fashion due to the change in glucose. A functional relationship between μ and an essential compound's concentration, in this case glucose, was proposed by Monod in 1942, which states:

$$\mu = \frac{\mu_{max} S}{K_S + S}$$

Here μ_{max} is the maximum achievable growth rate when the substrate, $S \gg K_S$ and the concentrations of all other essential compounds are unchanged. K_S is the value of the limiting nutrient concentration when $\mu = \frac{1}{2}\ \mu_{max}$.

Defining a yield coefficient, $Y_{x/s}$, can link substrate utilisation and cell growth.

$$Y_{x/s} = \frac{\Delta x}{\Delta S} = \frac{\mu x}{\Delta S / \Delta t}$$

where ΔS is the change in substrate concentration during cell growth and Δt is the time between t_0 and t_1. However, $Y_{x/s}$ is not a true constant and must be characterised for different culture environments. It is useful in estimating cell number where direct counting is not possible (e.g. immobilised cell cultures) and in optimising cell growth.

3.2.3 Semi-continuous culture of TEFLYRD/83

The ability of TEFLYRD/83 cells to continuously produce retrovirus particles was assessed in a semi-continuous culture over a 21 day period. Cells were inoculated into triplicate T-25 flasks at an initial density of 2×10^6 cells flask^{-1}. Pre-warmed culture

medium was added to each flask to give a final volume of 4.5ml. The cultures were inoculated at 37°C in a 5% CO_2 atmosphere for 3 days. The culture medium was changed and the total volume was reduced to 3ml per flask. The cultures were incubated for a further 2 days. A complete medium change was performed and the total medium volume was reduced to 1.5ml per flask. The medium was subsequently harvested daily and 1ml of the supernatant was stored in liquid nitrogen for later virus titration using the LacZ titration assay. The residual glucose concentration was measured and the glucose uptake rate was calculated. The total medium volume was increased when necessary to reduce the potential for substrate limitation. At the end of the experiment cells were detached from the flasks by trypsin and counted as described in chapter 2.

3.2.4 Semi-continuous culture of TEFLYRD/83 at 32°C

The performance of TEFLYRD/83 cells with regards to cell growth and virus production at 32°C was assessed in a semi-continuous culture. 5 x 10^6cells were inoculated into T-75 culture flasks with 13.5ml of DMEM supplemented with 10% FCS. These were incubated at 37°C with 5% CO_2 for 3 days. The medium was removed and replaced with 9 ml of fresh medium and the temperature of the incubator was reduced to 32°C. The flasks were left for 2 days to adapt to the lower temperature. After this the media was completely removed and replaced with 4.5ml of fresh media. A complete medium change was performed every 24 hours for a period of 10 days. The glucose concentration was measured daily in order to monitor cell viability. A 1ml sample of the harvested supernatant was stored in liquid nitrogen and the virus titre was later measured using the LacZ titration assay. At the end of the experiment cells were detached from the flasks using trypsin and counted using a

heamocytometer and microscope. Cell viability was determined using the trypan blue exclusion test.

3.2.5 Attachment and growth of TEFLYRD/83 on microcarriers

The attachment and growth of TEFLYRD/83 cells to different microcarriers was assessed and the final virus titre was measured. The microcarriers used were Cytoline 1, Cytoline 2 (Amersham Pharmacia, Sweden), Immobasil FS™ (Ashby Scientific LTD., UK) and Fibra-Cel™ (Bibby-Sterilin, UK). 2ml of microcarriers (dry volume) were prepared as described in chapter 2 and placed into 100ml spinner flasks (Bellco™, USA). Prior to inoculation the equilibration medium was completely removed. 10ml of fresh culture medium was added containing exponentially growing cells at a concentration of 2 x 10^6cells ml^{-1} microcarrier volume. The flasks were placed in an incubator during the attachment phase and gently shaken every 30 minutes to obtain uniform distribution on the carriers. A sample of supernatant was taken every hour and the number of cells in suspension was counted under a microscope using a heamocytometer. This was used to calculate the approximate number of attached cells. After 4 hours, culture media was added to give a final volume of 20 ml. A sample of microcarriers was taken and the cell density was determined using MTT (see chapter 2). The flasks remained under static conditions overnight. Agitation was started after 24 hours, for stirred cultures, at 40 rpm in a stirring incubator. Samples of microcarriers were taken every day and the cell density was estimated using MTT. Supernatant samples were taken daily and the concentration of glucose and lactate were measured. Cultures were run over 5 days. A 1ml sample of supernatant was collected at the end of the culture and stored in liquid nitrogen for virus titration using RT-PCR.

3.3 Results and Discussion

3.3.1 MTT Calibration Curve

In order to evaluate viable cell numbers from the MTT assay the relationship between cell number and absorbance was determined. Figure 3.3.1.1 shows the calibration curve obtained from cell suspensions of known density.

The MTT assay is useful for estimating viable cell density in immobilised cell cultures where direct counting of cell numbers is not possible as trypsinisation is required to remove cells from the matrix. However, the assay is dependant on the ability of mitochondrial enzymes to convert MTT into blue formazan. Unless the activity of cells within the culture is constant, the amount of formazan produced per cell may be variable (Yamaji and Fukuda 1992). It should also be noted that the amount of metabolic activity varies throughout the cell cycle, which will affect the assay. Therefore, estimation of cell numbers from the MTT assay should only be used as an indication of a change in cell density or activity or as a comparison of cell numbers between cultures from the same batch of cells (McTaggart 2000).

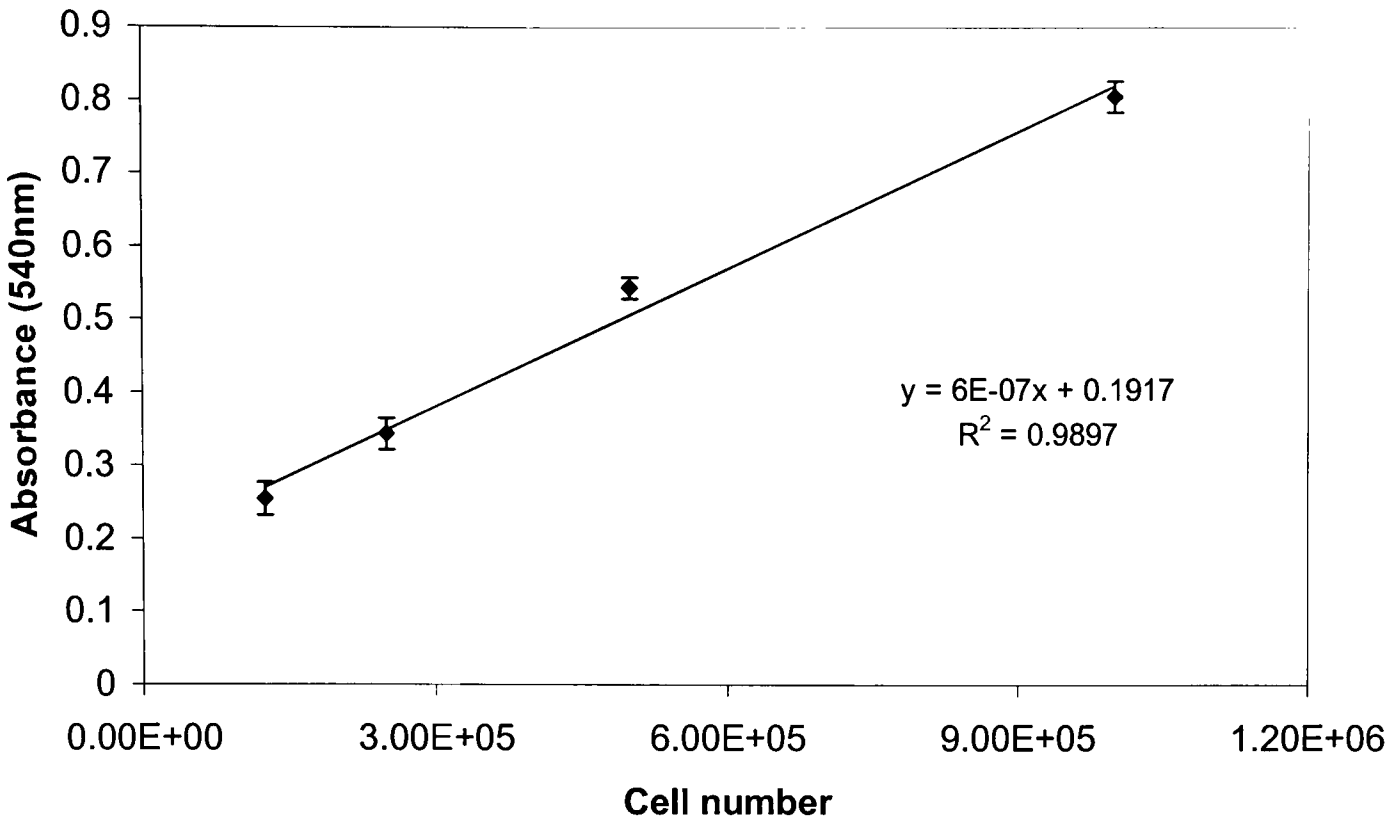


Figure 3.3.1.1

MTT calibration curve, showing the relationship between the TEFLYRD/83 cell number and the absorbance at 540nm. Known cell numbers were incubated with MTT for 2 hours before the formazan product was dissolved on acidic iso-propanol and the absorbance was measured at 540nm, using an SLT Spectra Elisa plate reader. Error bars represent standard deviation, n = 8.

3.3.2 Batch Culture of TEFLYRD/83 cells

Batch culture of TEFLYRD/83 cells was necessary to determine how the cells respond to changes in nutrient concentration and metabolic by-product accumulation. The growth curve obtained from the batch culture of TEFLYRD/83 cells followed the typical trend for mammalian cells. During the first day after initiation of the culture there was no increase in cell number, indicating the lag phase. The cell number increased in an exponential fashion until day 8, representing the log or exponential growth phase. The mean cell number had decreased after 10 days, indicating the start of the death phase at which point the experiment was ended. These results are illustrated in Figure 3.3.2.1.

Glucose consumption of TEFLYRD/83 cells was consistent during the first 8 days of the batch culture. By this time the mean concentration of residual glucose had dropped from 22.5mM L^{-1} to 0.65mM L^{-1}. This depletion in glucose coincides with the cells entering the death phase which suggests that glucose is the principal limiting substrate. Lactate production was consistent with glucose utilisation. The mean glucose and lactate concentrations in the culture supernatant are shown in Figure 3.3.2.2.

The yield coefficient $Y_{x/s}$ was calculated over the initial 8 days of the batch culture. This was deemed to be 2.18 x 10^3cells g^{-1} glucose. This value is useful for estimating cell numbers in monolayer cultures where direct counting is not possible and the metabolic demand of the cells is assumed to be constant with batch cultures.

The specific growth rate was calculated during the early exponential phase (after 1 day, up to 6 days) for TEFLYRD/83 cells. This was calculated as 0.484 day^{-1} which corresponds to a doubling time of 2.07 days. The instant specific growth rate was

determined and plotted against the glucose concentration, shown in Figure 3.3.2.3. The maximum specific growth rate was determined as 0.864 day^{-1} achieved when glucose was >19mM L^{-1}. The maximum specific growth rate was higher than values reported for CHO cells and PA317-RCM1 cells in batch culture (Leelavatcharamas *et al.* 1994;Wu *et al.* 2002), but lower than values reported for the FLYRD18 human packaging cell line (McTaggart and Al Rubeai 2001).

The mean virus titre increased for 6 days before a sharp decrease was observed (see Figure 3.3.2.4). The maximum titre achieved was 3 x 10^5LTU ml^{-1}. The virus titre reflects the changes in cell number, where a rapid increase in virus titre was seen during the exponential phase. The decrease in virus titre after 6 days may have been caused by two simultaneous events. Firstly, it has been previously reported that virus production is related to cell proliferation (Cruz *et al.* 2000; McTaggart and Al Rubeai 2001). After 6 days in batch culture the increase in cell number was slight, revealing that cell proliferation was diminishing. This reduction would result in a loss of virus production by the cell population. Secondly, as virus particles are unstable they will decay over time(Higashikawa and Chang 2001; Kaptein *et al.* 1997; Le Doux *et al.* 1999). The combination of reduced production and virus decay accounts for the dramatic decrease in virus titre during the closing 4 days of the batch culture.

It may be possible to overcome the problem of the loss in infectious virus particles by harvesting the supernatant when the cells reach the end of the exponential phase, and the virus titre is at its maximum. Alternatively, in order to avoid down time between batch cultures and a break in production as cells reach their maximum number, a semi-continuous culture could be performed. This has the advantage that cells are maintained at a constant number and daily medium replacement reduces the risk of nutrient limitation or the build up of toxic metabolites. While the cells are kept in an active metabolic state, virus particles can be produced and harvested daily, increasing

the overall virus titre for the experiment. A semi-continuous culture could be performed over an extended period of time e.g. >2 weeks. Once virus is collected it can be stored at a reduced temperature where it will be more stable than at 37°C. In addition to producing the viral vectors in a semi-continuous culture, the temperature may be reduced to 32°C to increase the virus stability in the culture supernatant. The possible disadvantage of reducing the temperature may be that the metabolic activity of the cells would decrease and virus production would lessen. These methods for increasing the overall productivity of culture systems are examined in the subsequent sections.

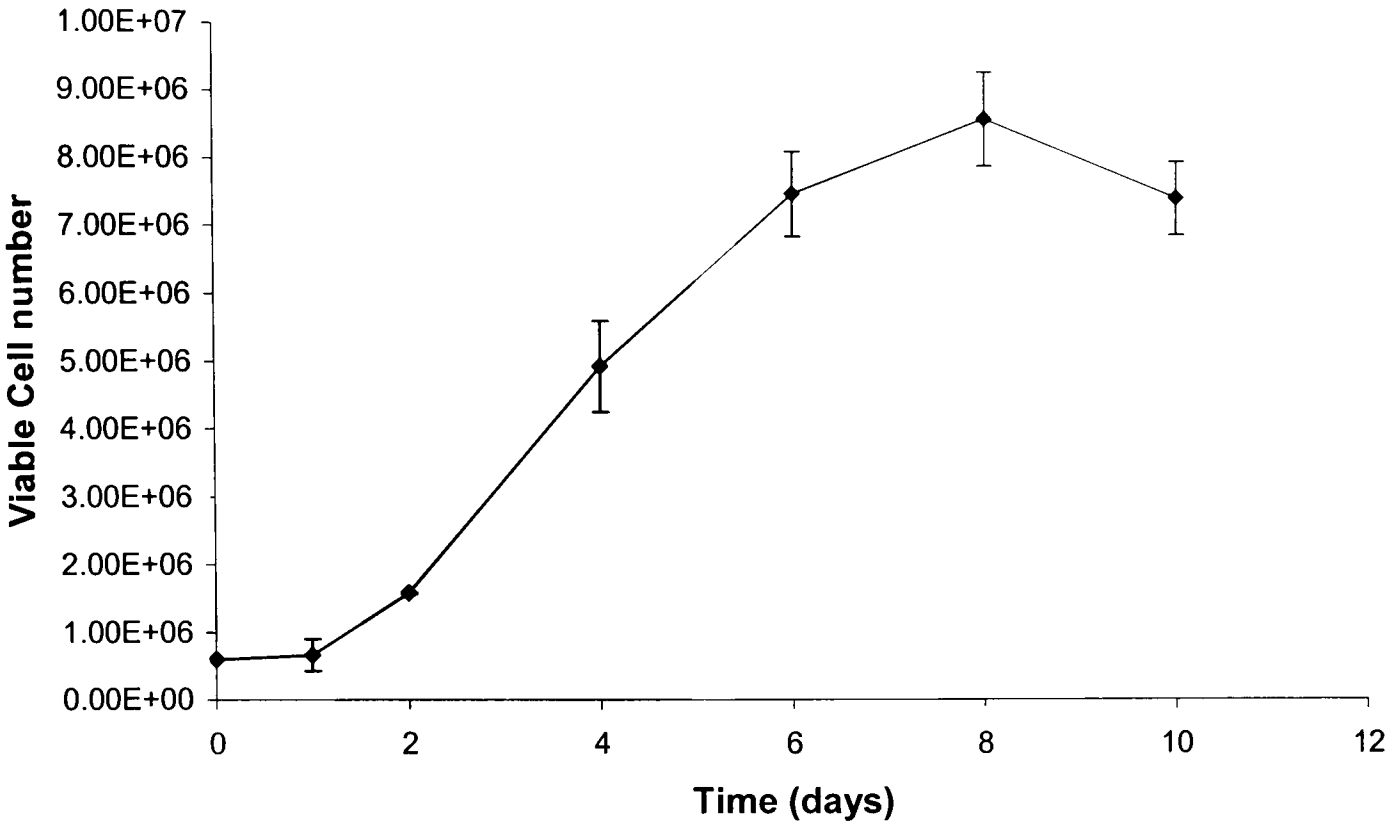


Figure 3.3.2.1

Mean viable cell number during a 10 day batch culture of TEFLYRD/83 cells in monolayer. Cells were inoculated into several $25cm^2$ T-flasks at a density of ~6×10^4 cells ml^{-1} in 10ml of culture medium, and incubated at 37°C in a 5% CO_2 atmosphere. Cells were detached from duplicate flasks at regular intervals and the viable cell number was counted using a haemocytometer under a microscope. Viability was determined by the trypan blue exclusion test. Error bars represent observed range, n = 2.

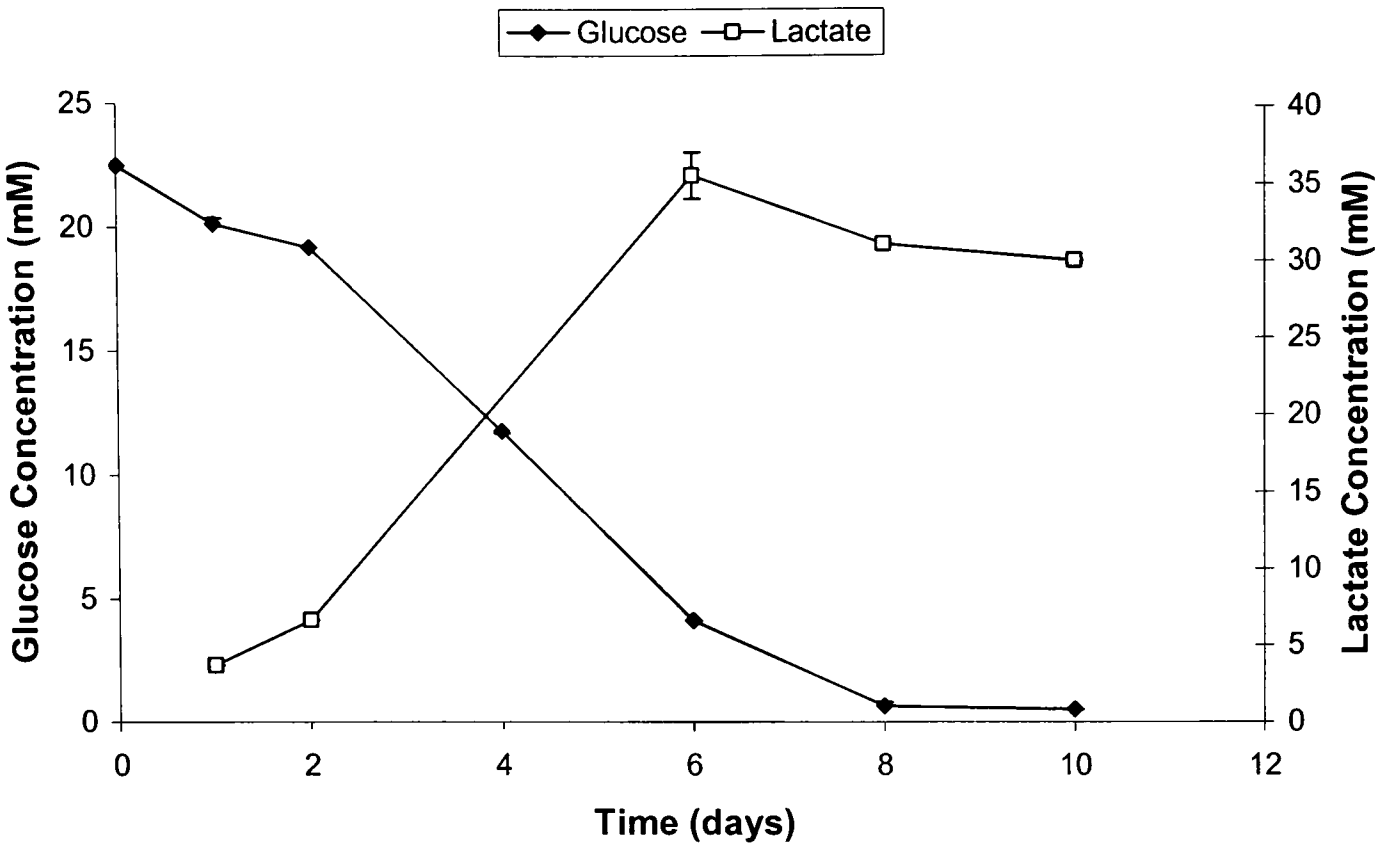


Figure 3.3.2.2

Mean residual glucose and lactate concentration during a 10 day batch culture of TEFLYRD/83 cells in monolayer. The supernatant was collected from duplicate flasks at regular intervals and the glucose was measured using the GLUCOTREND® glucose test kit (Roche, France) and lactate was measured using the Biolyser Rapid Analysis System (Eastman Kodak Co., USA). Error bars represent observed range, n = 2.

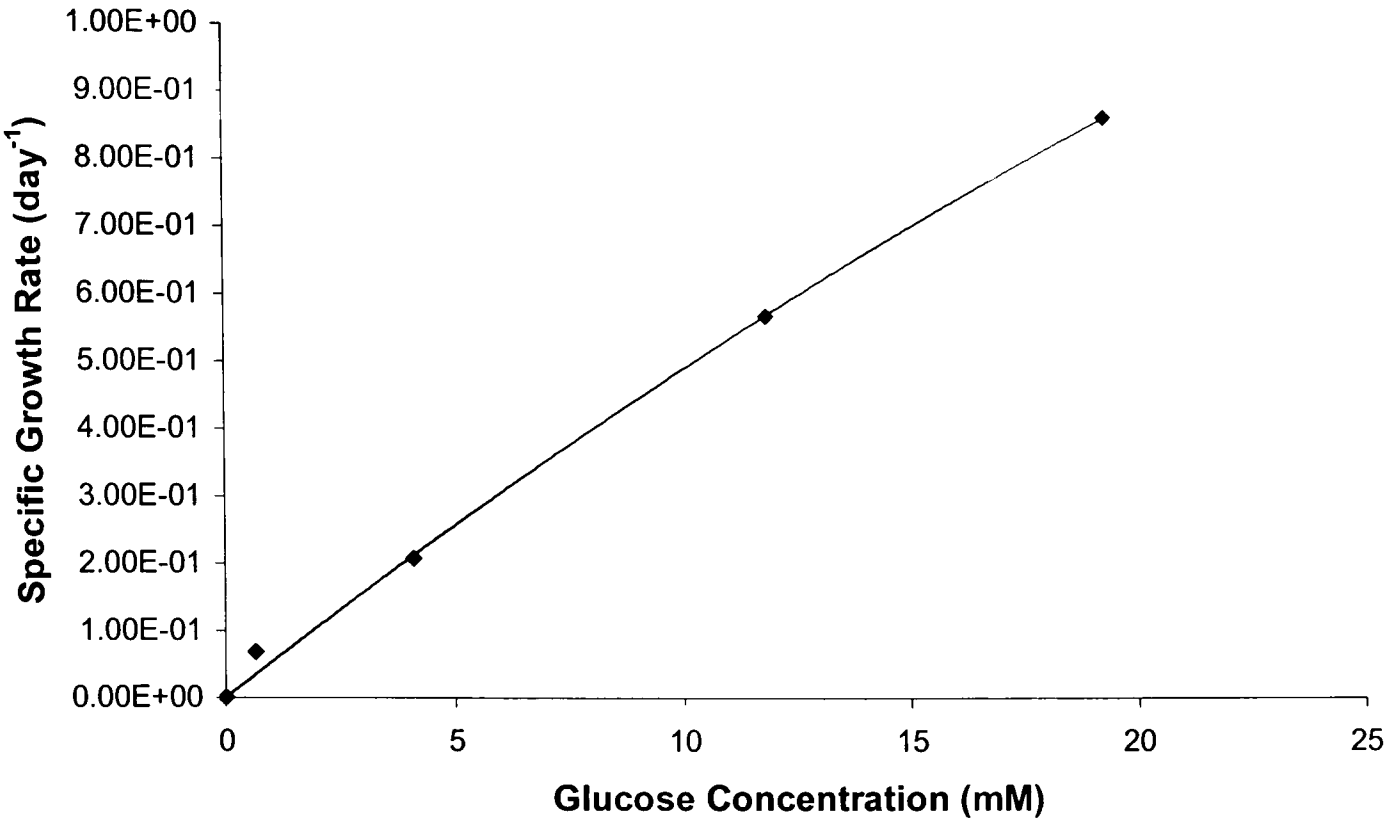


Figure 3.3.2.3

Effect of glucose concentration on specific growth rate of TEFLYRD/83 cells in batch culture. The instant specific growth rate was calculated by fitting the Monod equation (page 58) to the experimental data presented in Figure 3.3.2.1. The glucose concentration for each respective point was obtained from Figure 3.3.2.2.

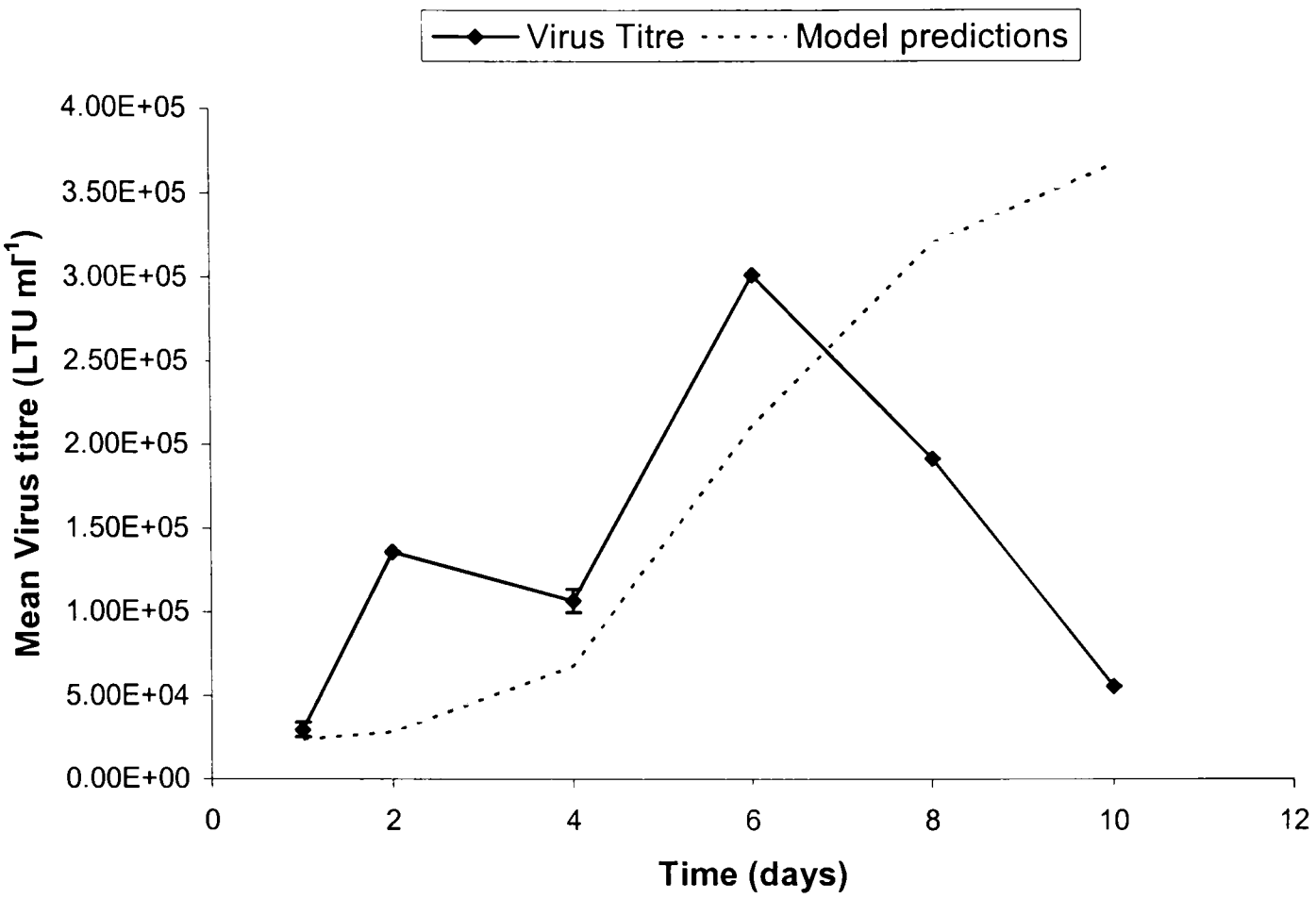


Figure 3.3.2.4

Mean daily virus titres during a 10 day batch culture of TEFLYRD/83 cells in monolayer. Virus containing supernatant was collected from duplicate flasks at regular intervals and stored in liquid nitrogen. The virus titre was later measured using the LacZ titration assay. The model presented in chapter 5 was used to predict virus titres. The model underestimates the infectious virus titre while cells are in the growth phase. However, it should be noted that this model is unable to account for possible changes in specific production rate due to changes in specific growth rate. Error bars represent observed range, n = 2.

3.3.3 Semi-continuous culture of TEFLYED/83

A semi-continuous culture of TEFLYRD/83 cells was performed in monolayer culture to determine its potential for virus production. Batch cultures have demonstrated that the cell line was capable of producing high virus titres but the number of infectious particles showed a dramatic decrease once cells entered the stationary growth phase. Daily harvesting of the supernatant with replacement of growth medium was thought to provide a means of overcoming the limitations observed in batch cultures.

The experiment was run for a total of 21 days. The final cell number from triplicate flasks was determined as $9.94 \pm 0.77 \times 10^6$ cells, which corresponds to a cell density of 3.97×10^6 cells ml^{-1} (see Figure 3.3.3.1).

The glucose uptake rate (GUR) and the residual glucose concentration are shown in Figure 3.3.3.2. After 6 days the residual glucose was completely utilised by the cells. It was therefore decided to increase the medium volume to 2.0ml. However, this was insufficient and the volume was increased to 2.5ml after 9 days, where it stayed for the remainder of the experiment. The constant GUR from day 10 onwards shows that cells had reached a constant number and that there was no decline in the total viable cell number. This demonstrates the ability of TEFLYRD/83 cells to survive over long periods of time and their suitability for long term production of virus particles.

The mean daily virus titres and the cumulative virus titre are recorded in Figure 3.3.3.3. During the production phase, where the culture medium was replaced daily, the mean virus titre showed some variance, unlike the assumed cell number. The mean virus titre increased up to day 10, as did the GUR. After 10 days, though, the mean virus titre began to decrease, whereas the GUR remained constant, with the exception of the final day when the virus titre showed an increase. If the cell number is constant, as can be assumed from the GUR, and the rate of virus decay is also

constant, as the culture conditions do not change, then the change in daily virus titre can be attributed to a change in cell specific virus productivity. The change in specific productivity is possibly due to the change in cell metabolism. After 10 days the cells reach the stationary phase, where the total cell number remains unchanged. Cells still proliferate but the number of cells produced is only equal to the number of cells that die so there is no net increase in cell number. As the cell population becomes established the amount of cell death may be reduced and therefore the cell proliferation will be reduced, leading to a decrease in virus production..

The cumulative virus titre was estimated to be 4.83 x 10^6 LTU. If it is assumed that during the same time period 3 batch cultures could be run then they would amass ~9 x 10^6 LTU. This number is based on the ability of each batch culture to produce 3 x 10^6 LTU (3 x 10^5 LTU ml^{-1}) over a six day period. The ability of batch cultures to produce a higher amount of vectors is due to the higher proliferative state of the cells as they are in the exponential growth phase during the initial six days of culture. In contrast, the maximum daily virus titre achieved in the semi-continuous culture was 1.62 x 10^5 LTU ml^{-1}. In addition to this, the batch cultures require a total of 30ml of culture medium compared to the semi-continuous cultures which utilised 45ml of culture medium. Although batch culture is more favourable for monolayer cultures this may not be true for larger scale cultures such as microcarrier cultures. In monolayer batch cultures a sufficient amount of medium is present to achieve a 10-fold increase in cell number. In microcarrier cultures a 100-fold increase in cell number may be required to maximise productivity and therefore medium replacement would be required. This experiment has shown the potential for long term cultivation of packaging cells and their ability to continually produce infectious virus particles.

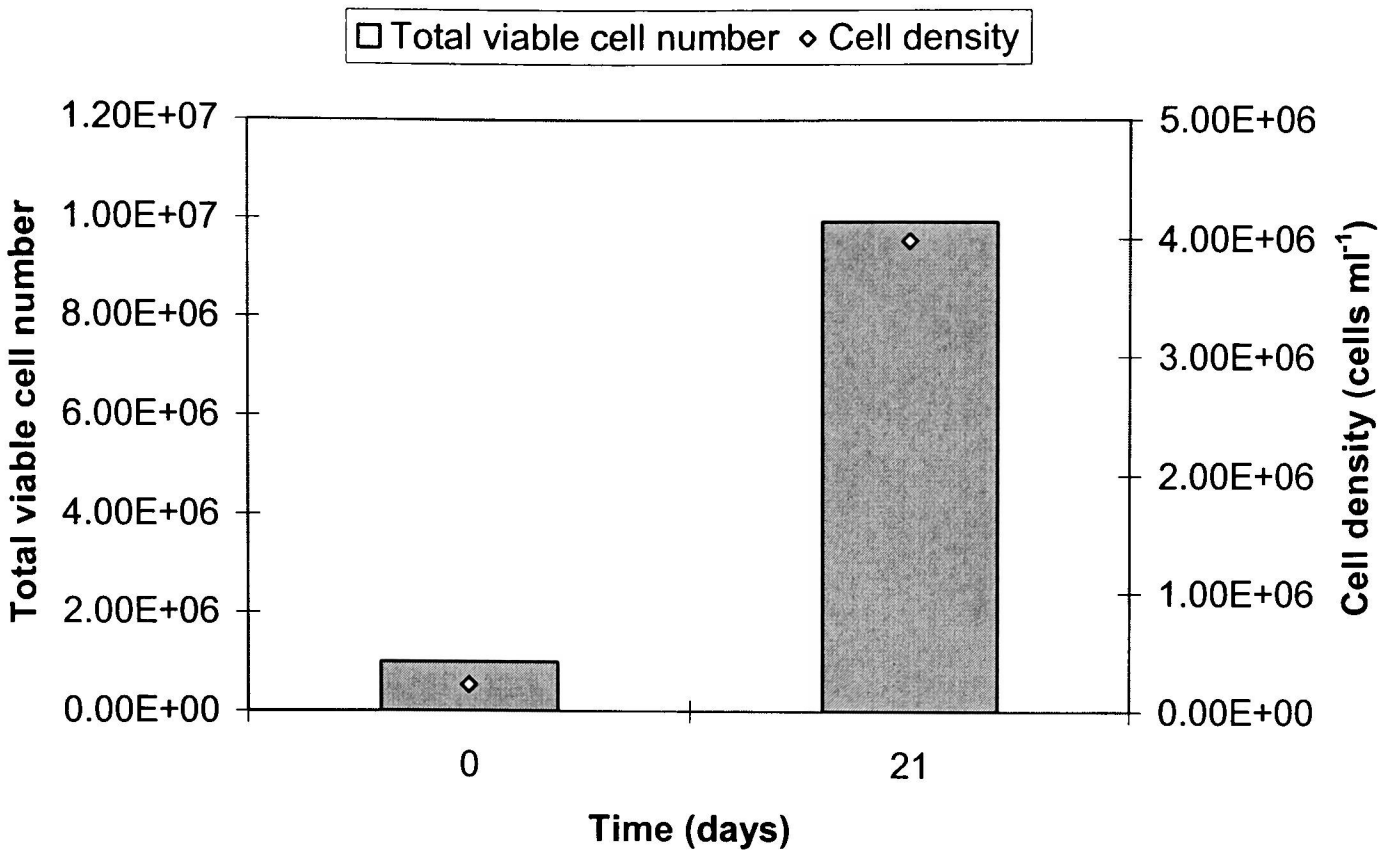


Figure 3.3.3.1

The initial and final viable cell number and cell density in a semi-continuous culture of TEFLYRD/83 cells in monolayer at 37°C. Cells were seeded into triplicate $25cm^2$ T-flasks at a concentration of 2×10^6 cells $flask^{-1}$. 4.5ml of medium was added to each flask before incubating at 37°C in a 5% CO_2 atmosphere. After 3 days the medium volume was reduced to 3ml, and then to 1.5ml after 5 days. To reduce the chance of glucose limitation the volume of medium was increased to 2ml after 6 days and then to 2.5ml after 9 days. The cell number was determined at the end of the experiment by detaching the cells from the flask surface by trypsin digestion and counting viable using a haemocytometer under a microscope. Viable cell numbers were determined by the trypan blue exclusion test. Direct measurement of cells was not possible during the course of the experiment as cells were attached to the flask surface.

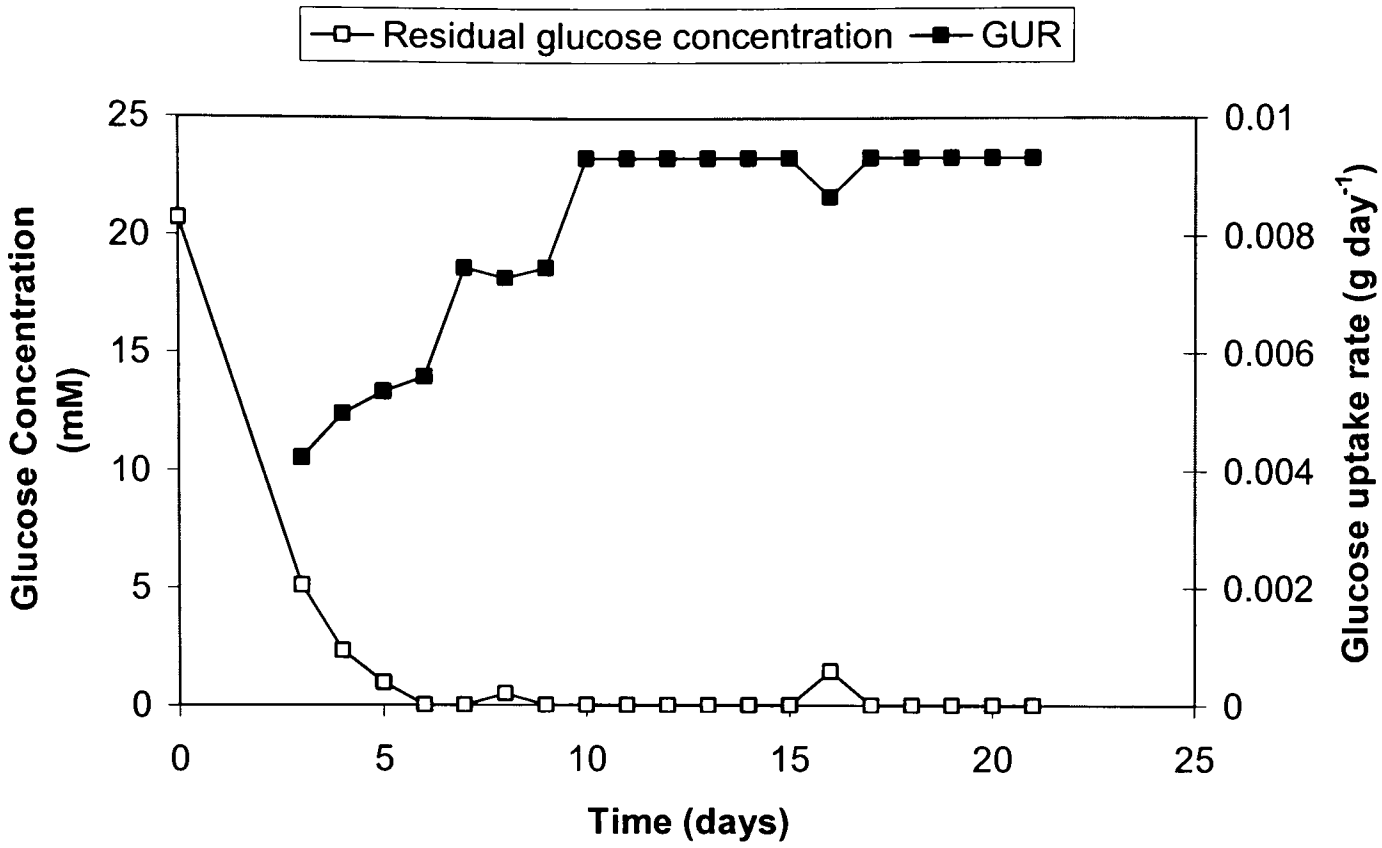


Figure 3.3.3.2

The Glucose uptake rate (GUR) and residual glucose concentration in a semi-continuous culture of TEFLYRD/83 cells in monolayer at 37°C. The initial medium volume was 4.5ml. After 3 days this was reduced to 3ml and then to 1.5ml after 5 days. The medium was changed daily from this point onwards. After 6 days the volume was increased to 2ml as all the glucose was fully utilised. Again, after 9 days the medium volume was increased to 2.5ml. The GUR was used as an indirect measurement of viable cell number and to monitor cell growth.

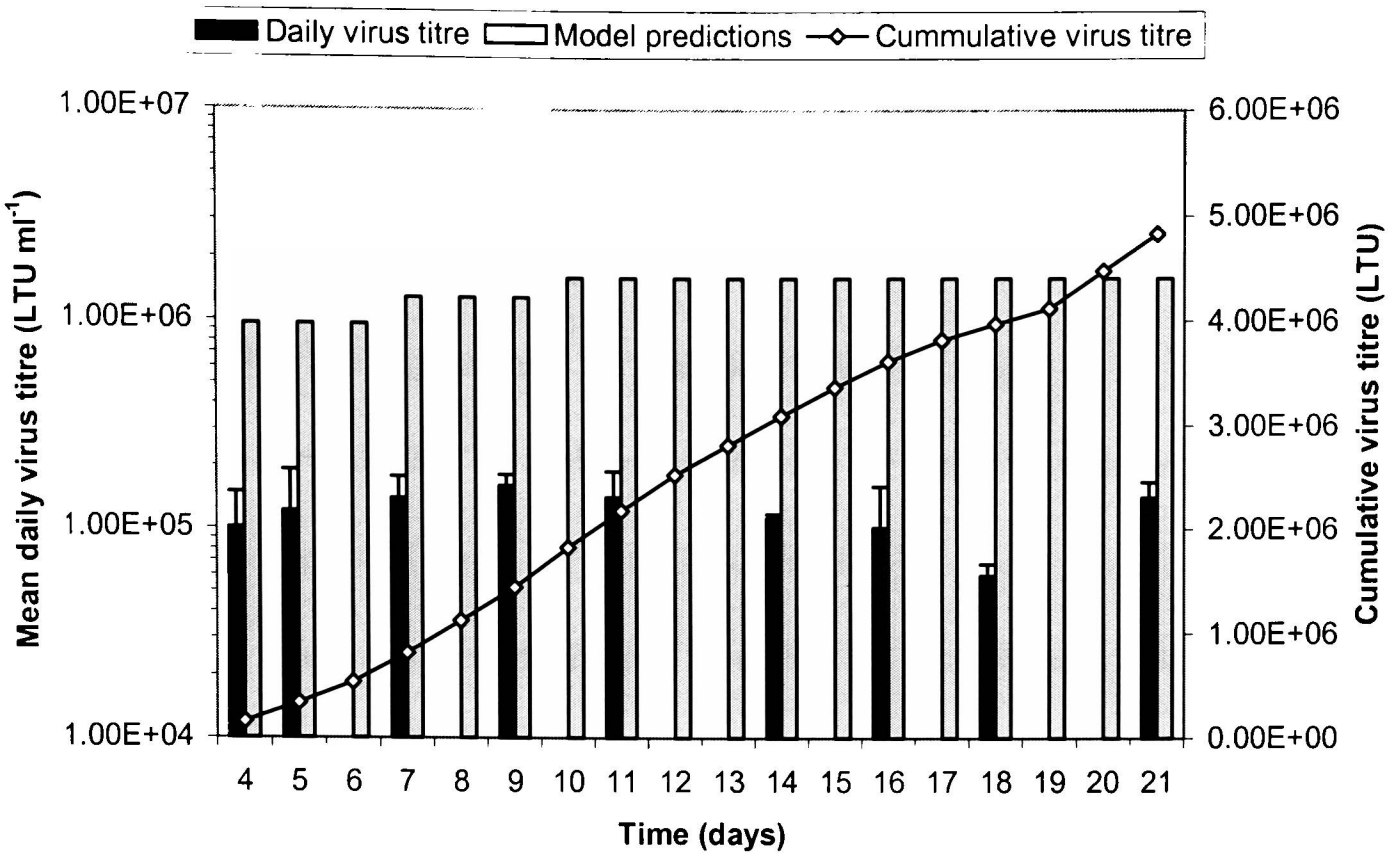


Figure 3.3.3.3

Mean daily virus titre and cumulative virus titre for a semi-continuous culture of TEFLYRD/83 cells in monolayer at 37°C. From day 4 onwards the culture medium was changed every day and the virus titres measured 3 times per week. The cumulative virus titre was estimated from median values of the titres recorded from the day immediately before and after. Virus titres were measured using the LacZ titration assay. The model presented in chapter 5 was used to predict daily virus titres. The predicted titres were 10-fold higher than those obtained. This demonstrates that the mode of bioreaction will influence the specific productivity. Error bars represent observed range, n = 2.

3.3.4 Semi-continuous culture of TEFLYRD/83 at 32°C

It is a well established fact that the virus decay rate is reduced at lower temperatures(Cruz *et al.* 2000; Higashikawa and Chang 2001; Kaptein *et al.* 1997; Le Doux *et al.* 1999; McTaggart and Al Rubeai 2000; McTaggart and Al Rubeai 2002). In order to improve the overall productivity of semi-continuous cultures, the temperature was reduced from 37°C to 32°C for the production phase of the culture. The total viable cell number decreased over the 15 day culture. However, the cell density increased due to the fact that the volume of medium was reduced from 13.5ml to 4.5ml. The initial and final cell numbers and densities are shown in Figure 3.3.4.1. The glucose concentration was measured daily to monitor the cell growth and viability. The glucose concentration and the glucose uptake rate are shown in Figure 3.3.4.2. There was no significant change in the daily glucose consumption rate during the 10 day production phase at 32°C. This suggests that the cell number was constant throughout. The glucose concentration did not fall below 10mM L^{-1} during the experiment which shows that there was no glucose limitation. The final cell density reached was 7.6×10^5 cells ml^{-1} compared to 3.98×10^6 cells ml^{-1} at 37°C. Therefore, at 32°C growth was limited by some factor other than glucose depletion as was the case in all previous experiments. The change in temperature would have an effect on the rate of cell metabolism. More than a thousand independent enzyme-catalyzed metabolic reactions occur in living cells. The rate constants of many enzyme-catalyzed reactions are dependent on temperature (Bailey and Ollis 1986). A reduction in temperature from 37°C to 32°C will result in a lower enzyme rate constant and hence slower cell metabolism, which accounts for the reduction in cell density.

The mean daily virus titres, like the glucose consumption, showed no significant variation over the course of the culture. The mean daily virus titre was 1.03 ± 0.31 x 10^6 LTU ml^{-1} day^{-1}, and was maintained for 10 days, as illustrated in Figure 3.3.4.3. The total cumulative virus titre was 4.64 x 10^7 LTU. The specific production rate, based on the final cell density and the virus titre recorded on day 15, was 0.71 LTU $cell^{-1}$ day^{-1}. The daily virus productivity showed a 10-fold increase compared to semi-continuous culture at 37°C.

As cell metabolism is slower at lower temperatures the virus production rate would also be expected to be lower. However, the reduction in temperature would increase the stability of virus particles, which explains the increased titres. The total amount of virus particles produced was significantly higher than all previous cultures, even when the difference in culture volume is taken into account. Therefore, a semi-continuous culture with a cell growth phase at 37°C and a vector production phase at 32°C appears to support the highest productivity for monolayer cultures. This mode of operation can be easily transferred to alternative methods of production.

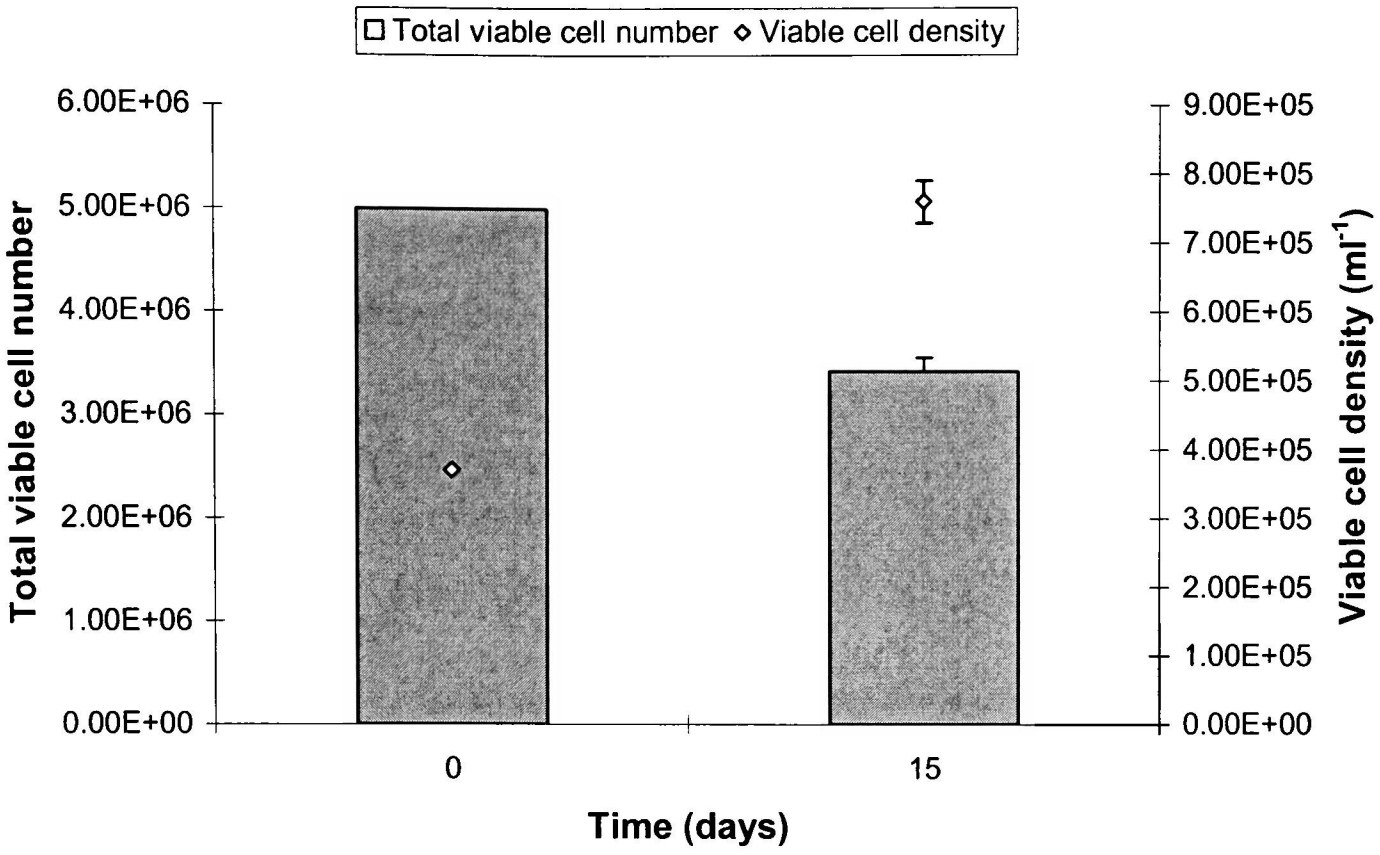


Figure 3.3.4.1

The initial and final viable cell number and density in a semi-continuous culture of TEFLYRD/83 cells in monolayer at 32°C. Cells were seeded into duplicate 75cm^2 T-flasks at a concentration of 5 x 10^6 cells flask^{-1}. 13.5ml of medium was added to each flask before incubating at 37°C in a 5% CO_2 atmosphere. After 3 days the medium volume was reduced to 9ml and the temperature lowered to 32°C. The medium volume was reduced to 1.5ml after 5 days and the medium was changed daily thereafter. The cell number was determined at the end of the experiment by detaching the cells from the flask surface by trypsin digestion and counting viable using a haemocytometer under a microscope. Viable cell numbers were determined by the trypan blue exclusion test. Direct measurement of cells was not possible during the course of the experiment as cells were attached to the flask surface. Error bars represent observed range, n = 2.

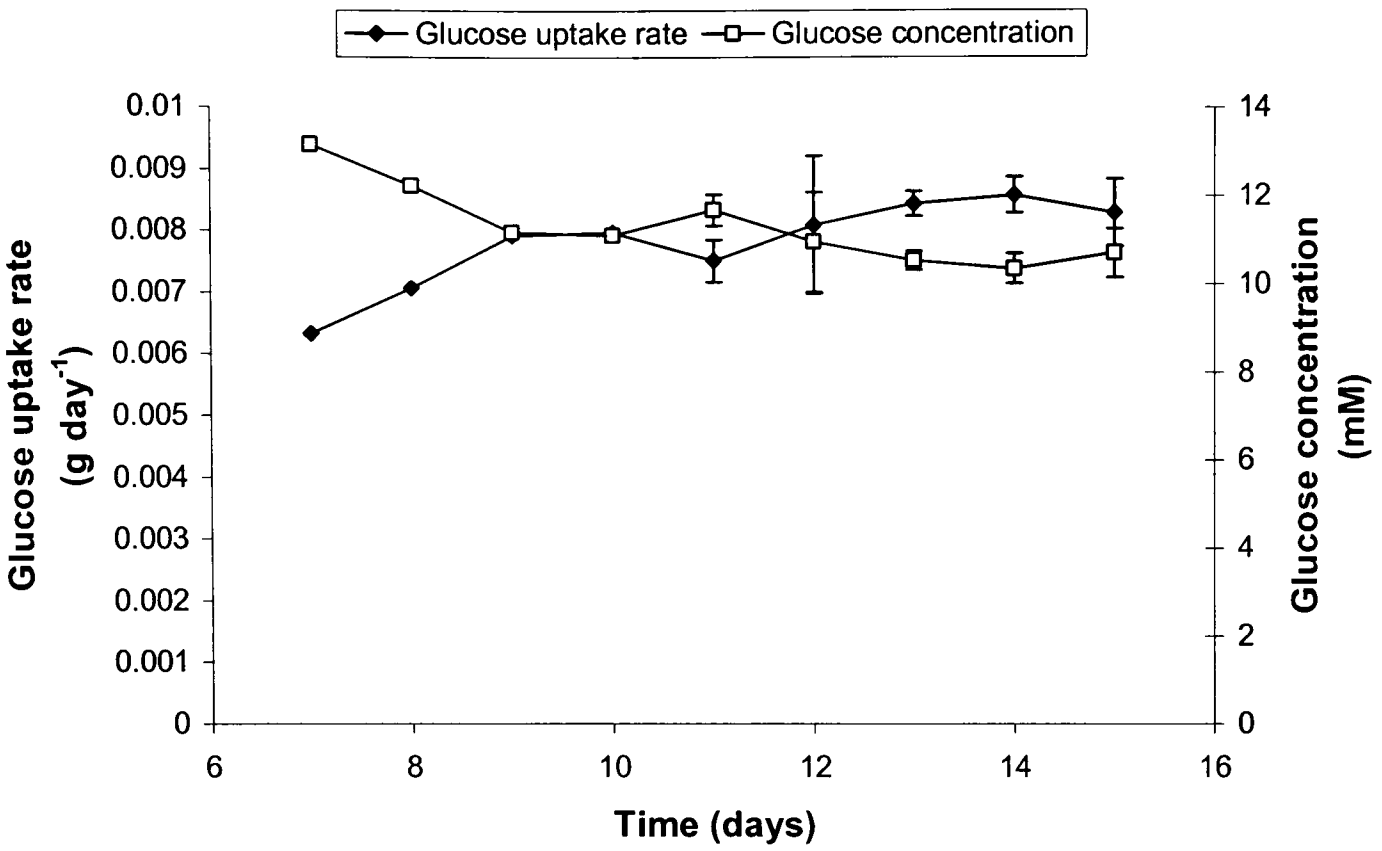


Figure 3.3.4.2

Glucose uptake rate and residual glucose concentration in a semi-continuous culture of TEFLYRD/83 cells in monolayer at 32°C. The initial medium volume was 13.5ml. After 3 days the temperature was reduced from 37°C to 32°C and the medium volume was reduced to 9ml and then to 4.5ml after 5 days. The medium was changed daily from this point onwards. The GUR was used as an indirect measurement of viable cell number and to monitor cell growth. Error bars represent standard deviation, n = 4.

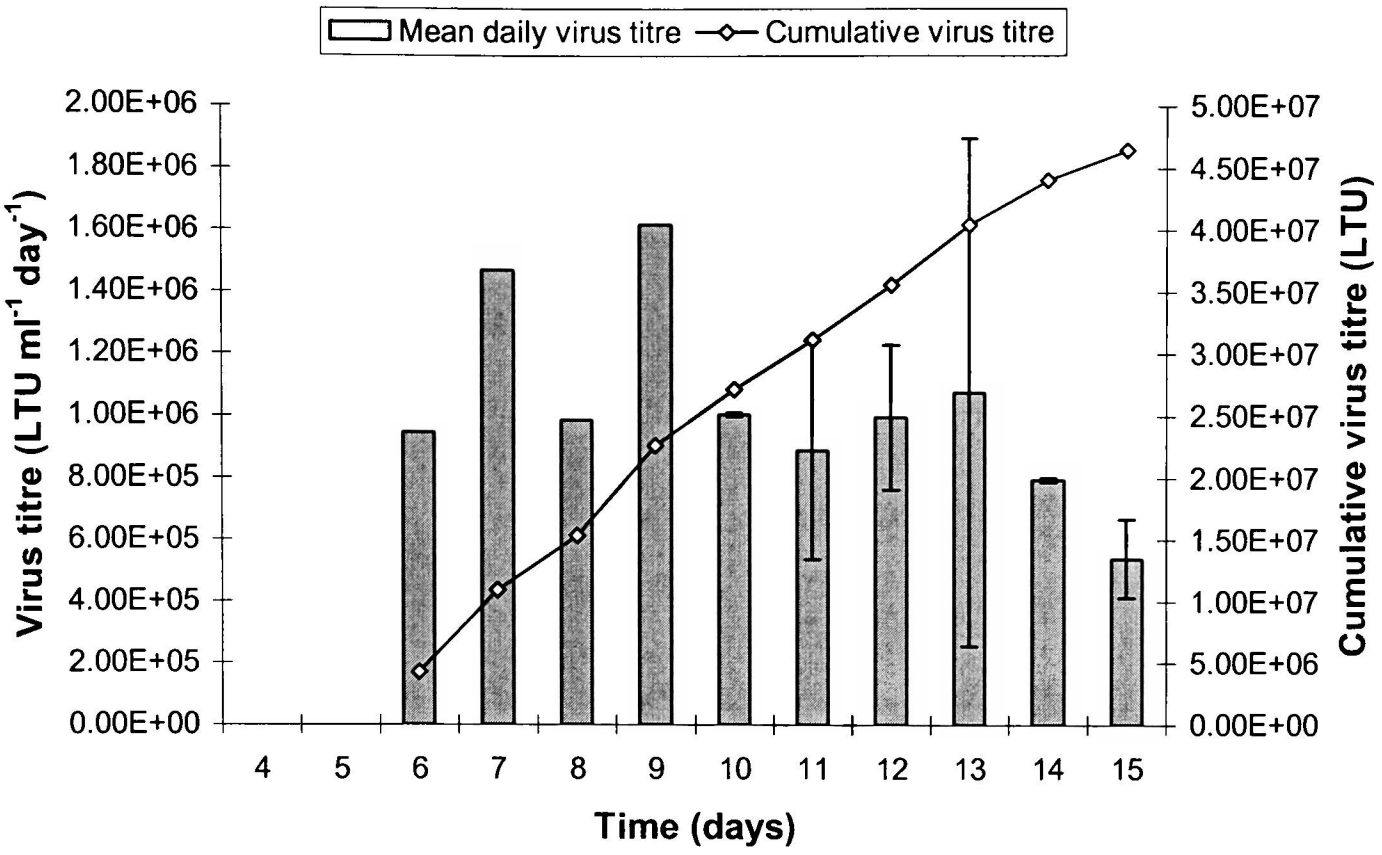


Figure 3.3.4.3

Mean daily virus titre and cumulative virus titre for a semi-continuous culture of TEFLYRD/83 cells in monolayer at 32°C. A complete medium change was performed from day 6 onwards. 1ml of the harvested supernatant was stored in liquid nitrogen until the end of the experiment. The virus titre was then measured suing the LacZ titration assay. Error bars represent standard deviation, n = 4.

3.3.5 Attachment and growth of TEFLYRD/83 on microcarriers

In order to facilitate the scale-up of retrovirus production, the available surface area for cell attachment and growth must be increased. Microcarriers offer the potential for high cell density cultures and can be used in a number of systems, such as stirred tank reactors, fluidised bed reactors and packed bed reactors.

Analysis of attachment kinetics

The rate of disappearance of cells from the supernatant follows an exponential decay curve, which is represented by:

$$x_t = x_0 \exp^{-kt}$$

where x_t is the cell concentration at time t, x_0 is the initial cell concentration and k is the attachment rate constant. This equation can be expressed logarithmically as:

$$-\ln(x_t / x_0) = kt$$

Consequently, a first-order rate would be represented by a straight line from a plot of $-\ln(x_t / x_0)$ vs. time with a gradient k. The value of k was interpreted as the specific attachment rate (hour^{-1}).

Evaluation of microcarriers for cell-bead attachment

In this study the rates of cell attachment to four different microcarriers, Cytoline 1 & 2, Immobasil FS and Fibra-Cel™, were examined under intermittent stirring modes by monitoring the disappearance of cells from the supernatant. In all cases the rate of bead attachment was rapid with >80% of cells attaching after 1 hour and >90% attaching after 2 hours. The most rapid attachment was seen with Immobasil FS microcarriers. There was no significant difference in the rate of attachment between

the other three carriers. The values of k were calculated from the data presented in Figure 3.3.5.1 by linear regression analysis and are given in Table 3.3.5.1.

A control experiment was performed, under identical conditions, without any microcarriers. The number of cells in suspension did not change significantly during the attachment phase, showing that cell disappearance from the medium was due to attachment to carriers and not to attachment to the vessel walls or cell lysis. Furthermore, the results from the MTT assay performed 4 hours post inoculation confirmed that cells had attached to the microcarriers. Cell attachment was observed by fluorescent microscopy and the results are depicted in Figure 3.3.5.2.

Cellular attachment occurs through electrostatic interactions (Ginsburg 1987) and charged surfaces support better cell attachment than carriers coated with an extracellular adhesion component such as collagen or fibronectin (Ng *et al.* 1996, Varani *et al.* 1998).The rapid attachment of cells to macroporous microcarriers is consistent with previous reports. It has been shown that the FLYRD18 cell line efficiently attaches to a variety of microcarriers within a few hours, and >80% of cells were able to attach to Immobasil FS carriers within 1 hour (Gerin *et al.* 1999a, McTaggart 2000). In contrast, it was reported by Merten *et al.* (2001) that TEFLY producer cells did not easily attach to solid microcarriers and had a tendency to form aggregates. The differences may be due to the surface properties of the carriers and the charge of the substrate; Cytodex solid microcarriers carry a high positive charge due to the N, N-diethylaminoethyl groups whereas the silica content of Cytoline and Immobasil carriers produces a slightly negative charge. Other factors that may effect cell attachment are the stirring mode (either continuous or intermittent), cell-bead ratio, bead concentration, culture pH or the concentration of serum (Mendonça *et al.* 1999, Ng *et al.* 1996, Shiragami *et al.* 1997).

Table 3.3.5.1

The attachment rate constant, k, of TEFLYRD/83 cells to different microcarriers under intermittent stirring modes in 100ml spinner flasks.

Microcarrier	Attachment rate constant, k ($hour^{-1}$)	r^2
Cytoline 1	1.3556	0.8898
Cytoline 2	1.3004	0.9730
Immobasil FS	1.7945	0.9554
Fibra-Cel™	1.3353	0.9546

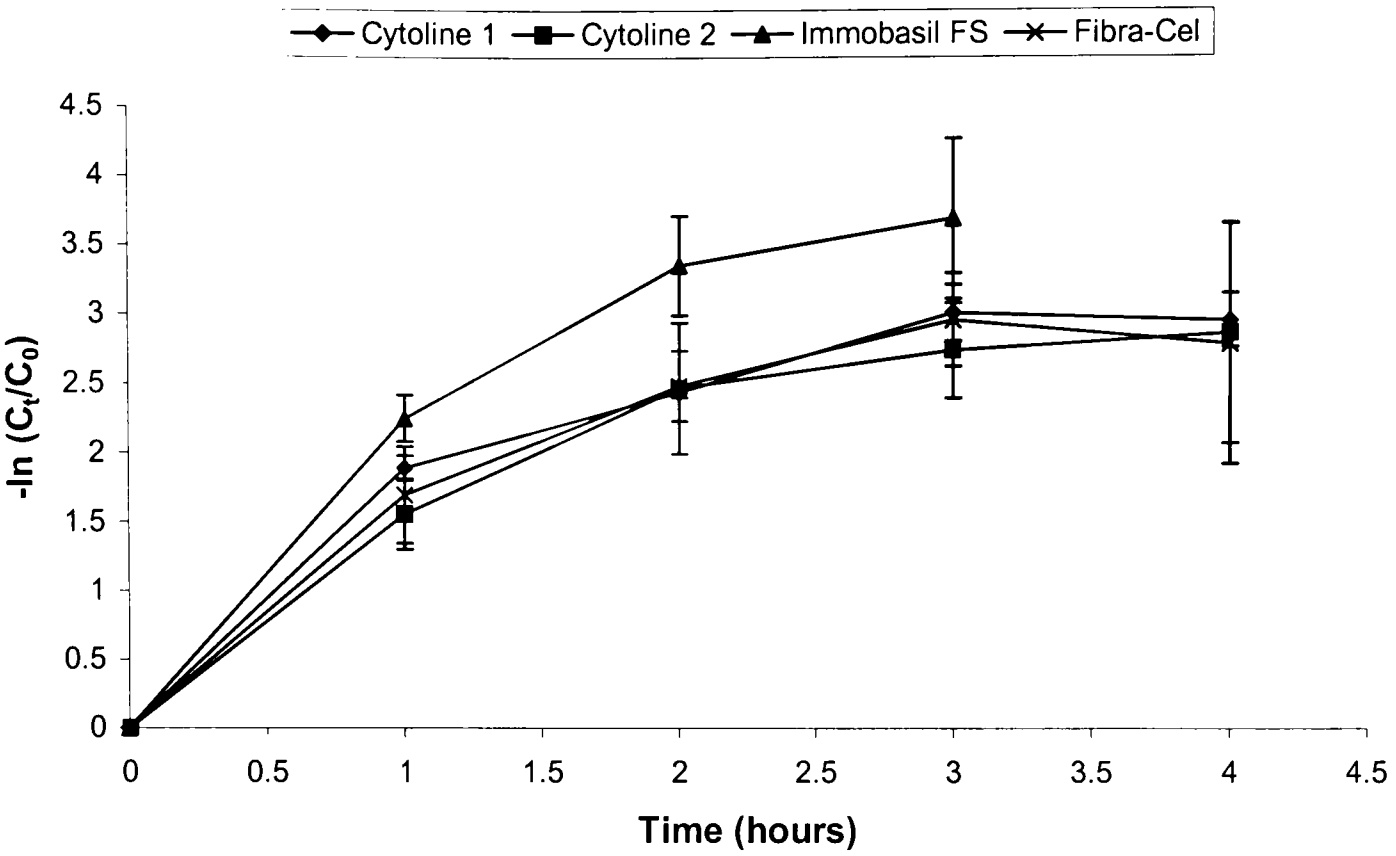


Figure 3.3.5.1

The rate of attachment of TEFLYRD/83 cells to different microcarriers. Cells were inoculated at a density of 2 x 10^6 cells ml^{-1} carrier in 10ml of culture medium. Culture vessels were 100ml spinner flasks containing 2ml of carriers. Cultures were stirred intermittently at 40rpm at 37°C and 5%CO_2. The number of attached cells was determined by counting the number of cells remaining in suspension and deducting this from the initial cell density. In a control study where no microcarriers were used, attachment of cells to the vessel walls, and no significant cell death was observed (results not shown). This implies that the disappearance of cells from suspension is due to attachment to the microcarriers. Error bars represent standard deviation, n = 4.

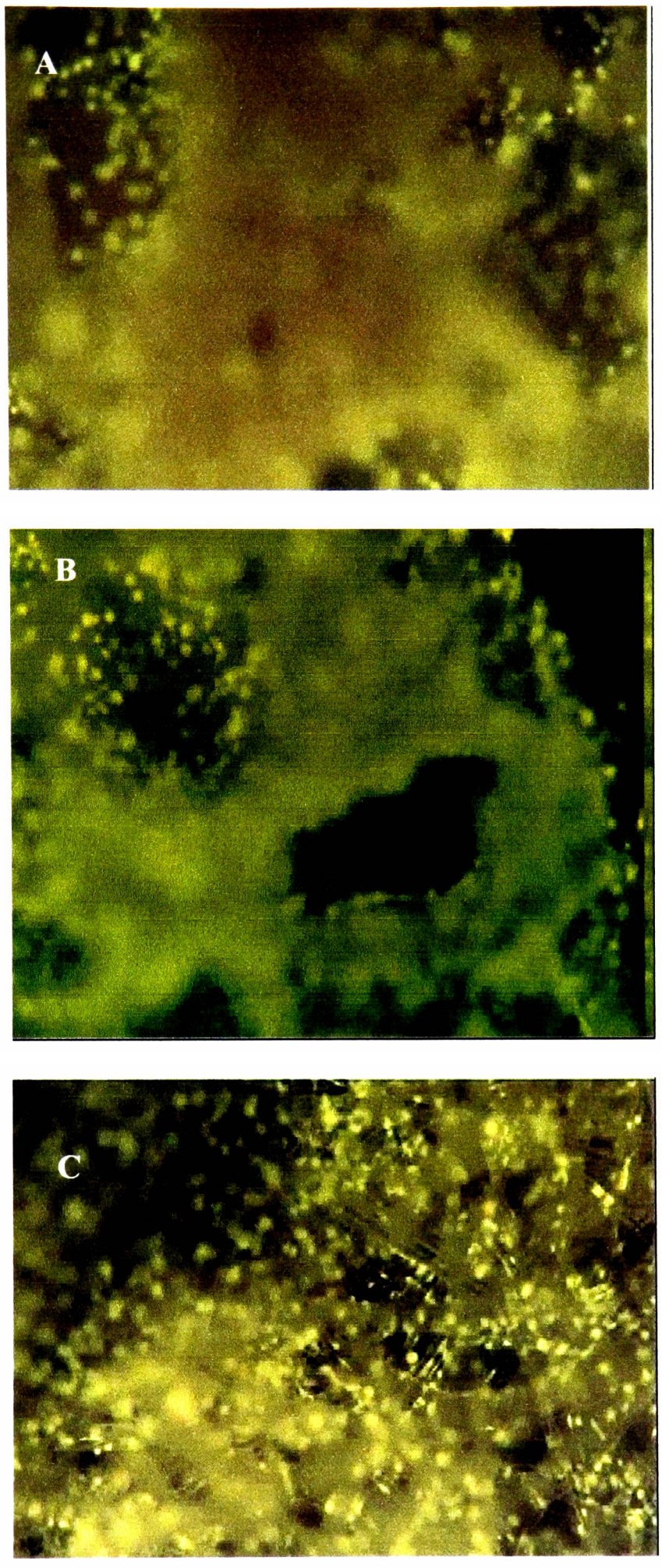


Figure 3.3.5.2

Observation of cell attachment to Cytoline 1 (A), Cytoline (B) and Fibra-Cel™ (C) microcarriers. Samples were stained with acridine orange and viewed under a fluorescent microscope 4 hours after inoculation.

Cell growth

Cell growth was observed under static and agitated conditions to determine whether or not cells could withstand the effects of hydrodynamic forces within stirrer flasks. In Kolmogorov's theory of isotropic turbulence it is hypothesised that the energy in large systems cascades down through ever decreasing eddies until it reaches such small scales that the energy is dissipated (Thomas and Zhang 1998). The scale is usually characterised by a wave number, K, which is inversely proportional to the eddy size. Hence, the largest wave number will give rise to the smallest eddies and this is determined by the effects of viscous dissipation. The Kolmogorov microscale of turbulence, η, is given by

$$\eta = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4}$$

where ν is the kinematic viscosity of the liquid and ϵ is the local energy dissipation rate. Therefore, the smallest eddy size is a function of the kinematic viscosity of the liquid and the local energy dissipation rate. A fluid-particle interaction is dependant on the relative sizes of the particles and eddies. If the eddies are larger than the particles they will simply entrain them and if the particle density is similar to the fluid there will be little relative motion and no damage. However, if the eddies are smaller than the particles they will be unable to entrain them resulting in a shear stress at the particle surface, possibly resulting in damage. This is an important consideration when using microcarriers as the particles are relatively big, with a diameter that ranges between 1mm for Immobasil FS and 6mm for Fibra-Cel™. The problem can be overcome by the fact that the beads have a macroporous structure. The cells will grow into these pores and will be protected from the hydrodynamic forces. However, during the early stages of the culture, immediately after attachment, cells will not have

had the chance to migrate into the pores and will be exposed to hydrodynamic forces on the microcarrier surface. For this reason, the length scale, η, was calculated for 100ml spinner flasks with 20ml of medium to assess the potential of using stirred vessels for production of retroviral vectors.

The culture medium was assumed to have the same kinematic viscosity and density as water. The kinematic viscosity of a microcarrier culture was estimated from(Croughan *et al.* 1987)

$$\nu = \frac{\mu_f\left(1+2.5\phi+10\phi^2\right)}{\rho_f(1-\phi)+\rho_s(\phi)}$$

where μ_f is the fluid viscosity (10^{-3} kg $m^{-1}s^{-1}$), ρ_f is the fluid density (10^3 kg m^{-3}), ρ_s is the solid (microcarrier) density, and ϕ is the volume fraction of solids (0.10). The values obtained are shown in Table 3.3.5.2.

The mean energy dissipation rate, ε_T was calculated from

$$\overline{\varepsilon_T} = \frac{PoN^3D^5}{V}$$

where Po is the power number, a function of impeller Reynolds number ($Re = ND^2/\nu$) and impeller configuration, N is the impeller speed, D is the impeller diameter and V is the culture volume. For Re > 1000, Po for most tissue culture systems may be considered constant with value 0.5 without major error (Cherry and Papoutsakis 1988). The values used to calculate the mean energy dissipation rate were Po = 0.5, N = 0.667rps, D = 0.051 m, and V = 2 x 10^{-5} m^3. The mean energy dissipation rate was therefore 2.56 x 10^{-3} m^2s^{-3}. Consequently, the Kolmogorov microscale of turbulence was calculated for each microcarrier and the results are given in

Table 3.3.5.2. The typical size of cells is 10-20μm and therefore the agitation will not cause any direct cellular damage. However, it is assumed that the interaction of eddies at the microcarrier surface will cause cell detachment, resulting in death. For this reason, the cultures were left for 24 hours post attachment to allow the cells to migrate into the microcarrier pores where they would be protected from surface detachment before stirring was started.

Table 3.3.5.2

Physical parameters of microcarrier cultures in 100ml spinner flasks containing 20ml of culture medium.

Microcarrier	Density, ρ_s ($g\ ml^{-1}$)	Kinematic viscosity, ν ($m^2\ s^{-1}$)	Microscale of turbulence, η (μm)
Cytoline 1	1.32	1.308×10^{-6}	172
Cytoline 2	1.03	1.346×10^{-6}	176
Immobasil FS	1.065	1.341×10^{-6}	175
Fibra-Cel™	1.04	1.345×10^{-6}	176

TEFLYRD/83 cells were grown on Cytoline 1 microcarriers in spinner flask cultures under static and stirred conditions. As seen from Figure 3.3.5.3, cells did not appear to have a lag phase under either condition. As expected the cell number was identical after 1 day in both sets of cultures as agitation had not begun. After 2 days the cell number in stirred flasks was slightly lower than in static flasks, although the difference in cell density was relatively small. After 3 days the cell density had shown a sharp decrease in both cultures. This shows that cell death had not been caused by agitation but in both cases a limiting factor was present. Glucose concentrations were measured (results not shown) and had not been exhausted. Therefore, some other limiting factor that had not been measured was present, such as low dissolved oxygen or glutamine concentration, or a build up in toxic metabolites such as lactate or ammonia, or a substantial decrease in pH due to CO_2 production.

Cell growth on Cytoline 2 carriers showed similar characteristics (see Figure 3.3.5.4). No lag phase was observed in the cultures and cell density was not significantly different after 1 day. The cell density in stirred cultures increased after 2 days but was not as great as in static cultures suggesting that agitation may have had an effect on cell growth. As with Cytoline 1 carriers, the cell density declined after 3 days in stirred cultures. However, cell density in static cultures did not change significantly during the following 2 days suggesting that cell growth was limited but the culture was able to maintain the cell density.

As with the Cytoline carriers, no lag phase was observed in cell growth on Immobasil FS microcarriers. Cell densities were similar after 1 day for stirred and static cultures. After 2 days the viable cell density in stirred cultures had fallen by approximately 80%, as can be seen in Figure 3.3.5.5. The cell density in static cultures had continued to increase, though. This suggests that cells had largely remained on the outside of the particles and had not grown into the pores. Hence, when agitation had begun a large

proportion of the cells had been detached from the carriers resulting in the decline in viable cell density. After this initial drop in cell density the cells recovered slightly but after 4 days the density was only about 50% of the density 4 hours after inoculation. Cells in static culture did increase in number over the course of the experiment and did not show any decrease although their growth rate decreased after day 3. This shows that cells can attach and grow on Immobasil FS carriers but they may not be suited to stirred cultures.

Cell growth on Fibra-Cel™ discs was not as rapid as the other beads, as illustrated in Figure 3.3.5.6. There was no significant difference in cell density between stirred and static cultures after 2 days and this only increased by 2-fold. After 3 days the cell density under both culture conditions had increased. The increase was greatest in stirred cultures. This trend lasted to the end of the experiment. This implies that agitation was necessary for nutrients to diffuse into the structure of the discs. Under static conditions the mass transfer to cells was slower resulting in a reduction in growth rate.

The highest cell density achieved was 2.06×10^7 cells ml^{-1} carrier, using Cytoline 2 microcarriers under static conditions. This was a ten-fold increase in cell number achieved after 3 days and showed a maximum specific growth rate of 1.075 day^{-1}. Under stirred conditions the maximum cell density achieved was 1.55×10^7 cells ml^{-1} carrier, using Cytoline 1 microcarriers. The specific growth rate was 0.820 day^{-1}. Fibra-Cel™ discs and Cytoline 2 beads had similar maximum cell densities of 1.16×10^7 cells ml^{-1} carrier and 1.23×10^7 cells ml^{-1} carrier, respectively, and their specific growth rates were 0.503 day^{-1} and 0.743 day^{-1}, respectively. Immobasil FS had the lowest cell density under stirred conditions, reaching 3.26×10^6 cells ml^{-1} carrier after 4 days.

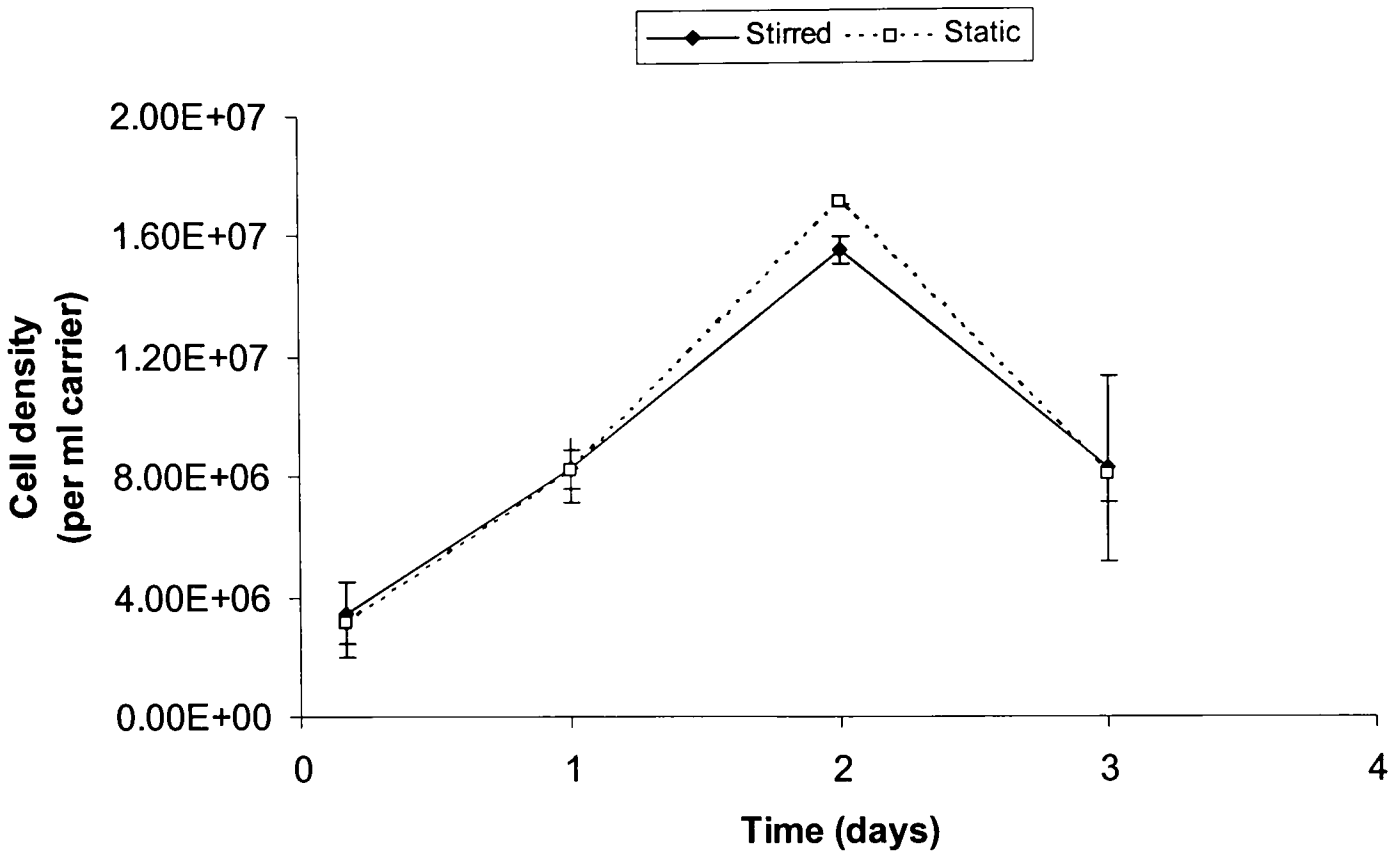


Figure 3.3.5.3

Mean viable cell number during a 5 day batch culture of TEFLYRD/83 cells in 100ml spinner flasks (working volume 20ml) on Cytoline 1 microcarriers under stirred (40rpm) and static conditions. The flask impeller had a diameter of 51mm. Error bars represent observed range, n = 2.

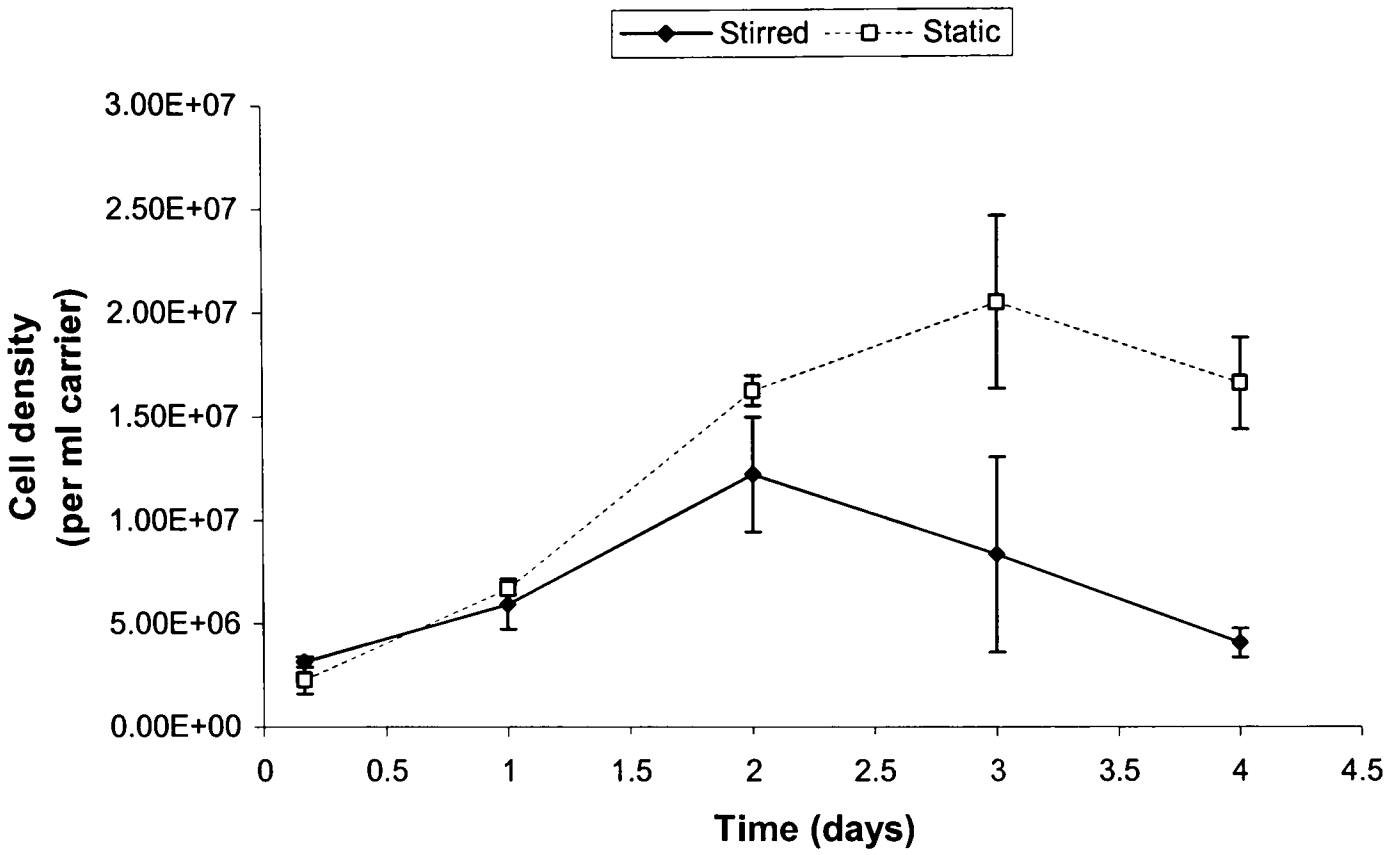


Figure 3.3.5.4

Mean viable cell number during a 5 day batch culture of TEFLYRD/83 cells in 100ml spinner flasks (working volume 20ml) on Cytoline 2 microcarriers under stirred (40rpm) and static conditions. The flask impeller had a diameter of 51mm. Error bars represent observed range, n = 2.

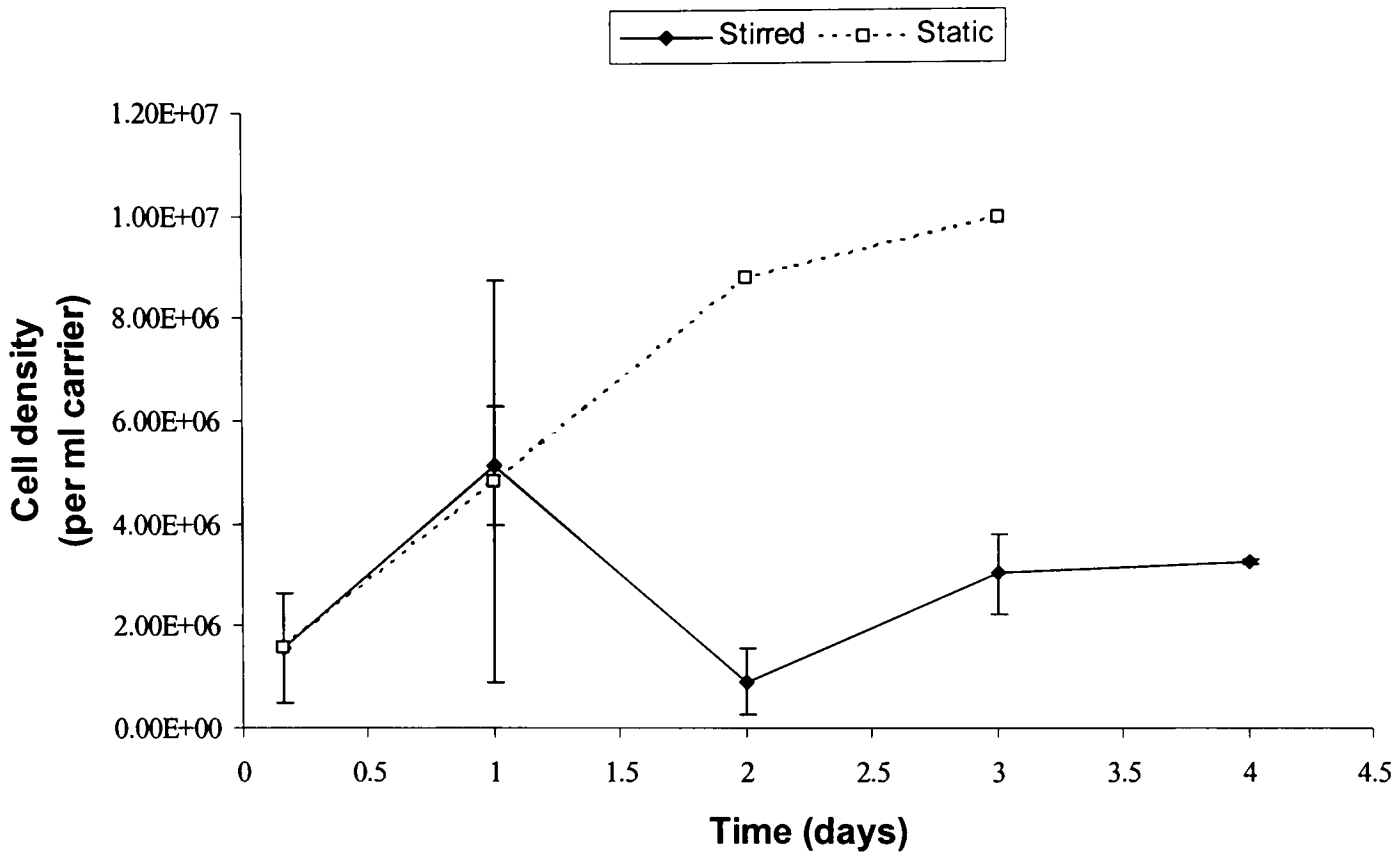


Figure 3.3.5.5

Mean viable cell number during a 5 day batch culture of TEFLYRD/83 cells in 100ml spinner flasks (working volume 20ml) on Immobasil FS microcarriers under stirred (40rpm) and static conditions. The flask impeller had a diameter of 51mm. Error bars represent observed range, n = 2.

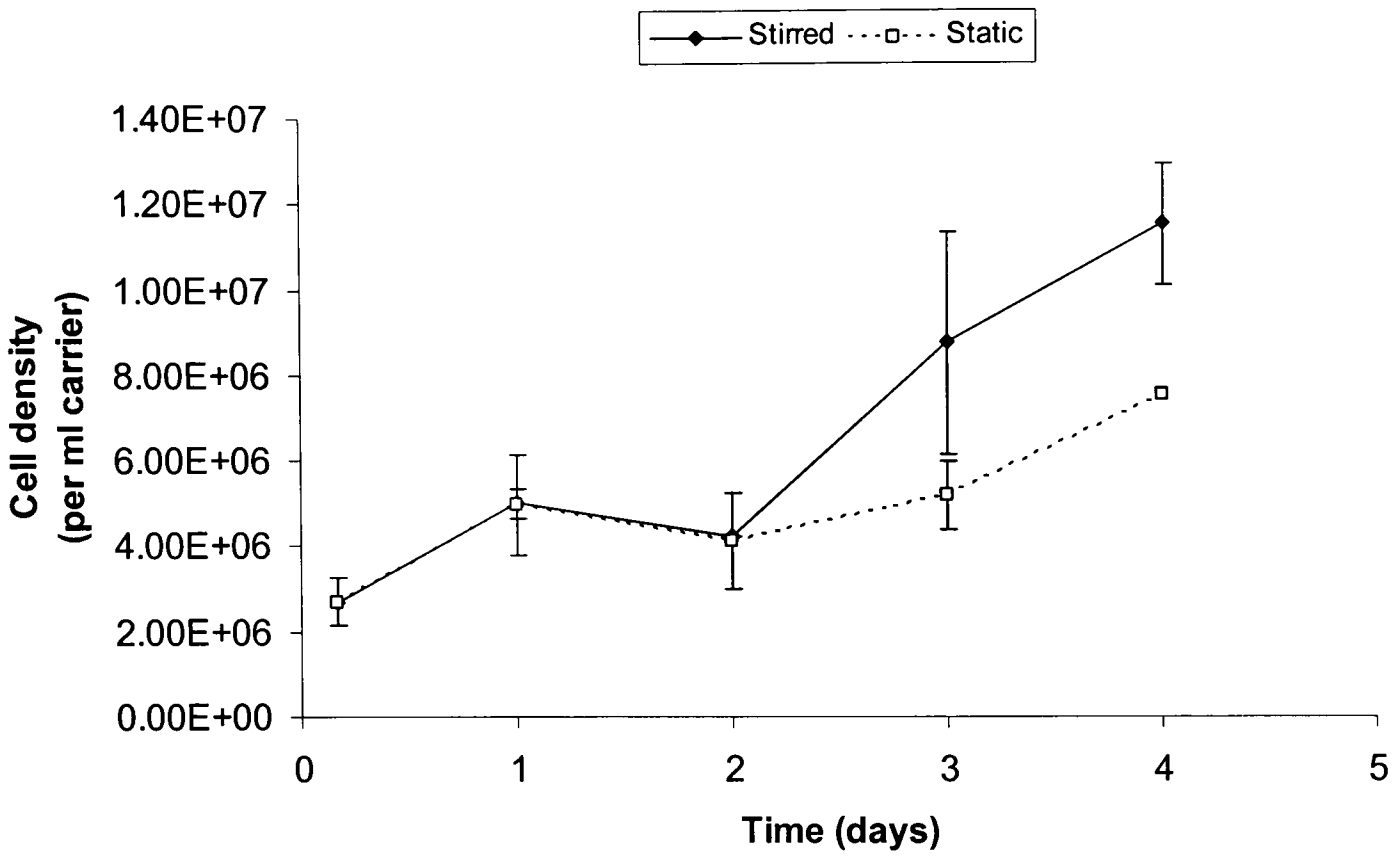


Figure 3.3.5.6

Mean viable cell number during a 5 day batch culture of TEFLYRD/83 cells in 100ml spinner flasks (working volume 20ml) on Fibra-Cel™ microcarriers under stirred (40rpm) and static conditions. The flask impeller had a diameter of 51mm. Error bars represent observed range, n = 2.

Virus production

On the final day of the experiment, samples of the supernatant were taken from each spinner flask and the virus titre was measured using RT-PCR. The results are presented in Figure 3.3.5.7. No significant difference was seen between stirred and static cultures, which imply that agitation has no affect on virus production. As shown in, the microscale of turbulence in spinner flask cultures was ~175 μm. Therefore, there would be no interaction between the fluid and the cell surface during viral budding, and thus no damage or premature cleavage of virus particles. Once the virus particles were in suspension they would not be damaged by eddies but entrained by them.

The virus titres obtained from Cytoline 1 and 2 were much smaller than those obtained from Immobasil FS or Fibra-Cel™. This can be attributed to the fact that the cell number peaked after 2-3 days for these cultures. As can be seen from batch cultures of TEFLYRD/83 in section 3.3.2, when the cells enter the death phase the virus titre shows a significant decrease. After 4 days the level of virus production from these cultures would be greatly reduced and it is expected that the titres would be lower than for Immobasil FS or Fibra-Cel™ where the cells were actively growing. This is reflected in the apparent specific production rate.

The apparent specific production rate was calculated based on final cell number and the virus titre. The results are presented in Table 3.3.5.3. The results obtained are higher than those reported by Cruz *et al.* (2000), who produced retrovirus from PA317 cells, using Cultisphere-S macroporous microcarriers in 125ml stirred cultures. The virus titres are also higher than those recorded by Gerin *et al.* (1999a), where the FLYRD18 cell line was cultivated under similar conditions but using Cultisphere-G and Cytopore 1 microcarriers. This suggests that the TEFLYRD packaging cell line is

well suited to microcarrier culture. The productivity was higher in stirred cultures than static cultures for all microcarriers. A common problem with macroporous microcarriers is that virus particles are unable to enter the free supernatant to be harvested. Instead they remain within the matrix where they decay, leading to an overall reduction in productivity. Hence, virus production may be higher from solid microcarrier cultures even though cell densities are lower than in macroporous cultures (Berry *et al.* 1999, Wu *et al.* 2002). The higher productivity observed in agitated cultures may not be due to increased cell specific production but an improvement in product transport into the free supernatant. Therefore, intraparticle fluid flow is an important factor in the design of any virus production system.

Table 3.3.5.3

The apparent specific production rate for TEFLYRD/83 cells in different microcarrier cultures under static and stirred conditions.

Microcarrier	Specific production rate under static conditions (LTU $cell^{-1}$)	Specific production rate under stirred conditions (LTU $cell^{-1}$)
Cytoline 1	0.601	0.686
Cytoline 2	0.098	0.434
Immobasil FS	3.683	8.670
Fibra-Cel™	4.589	4.726

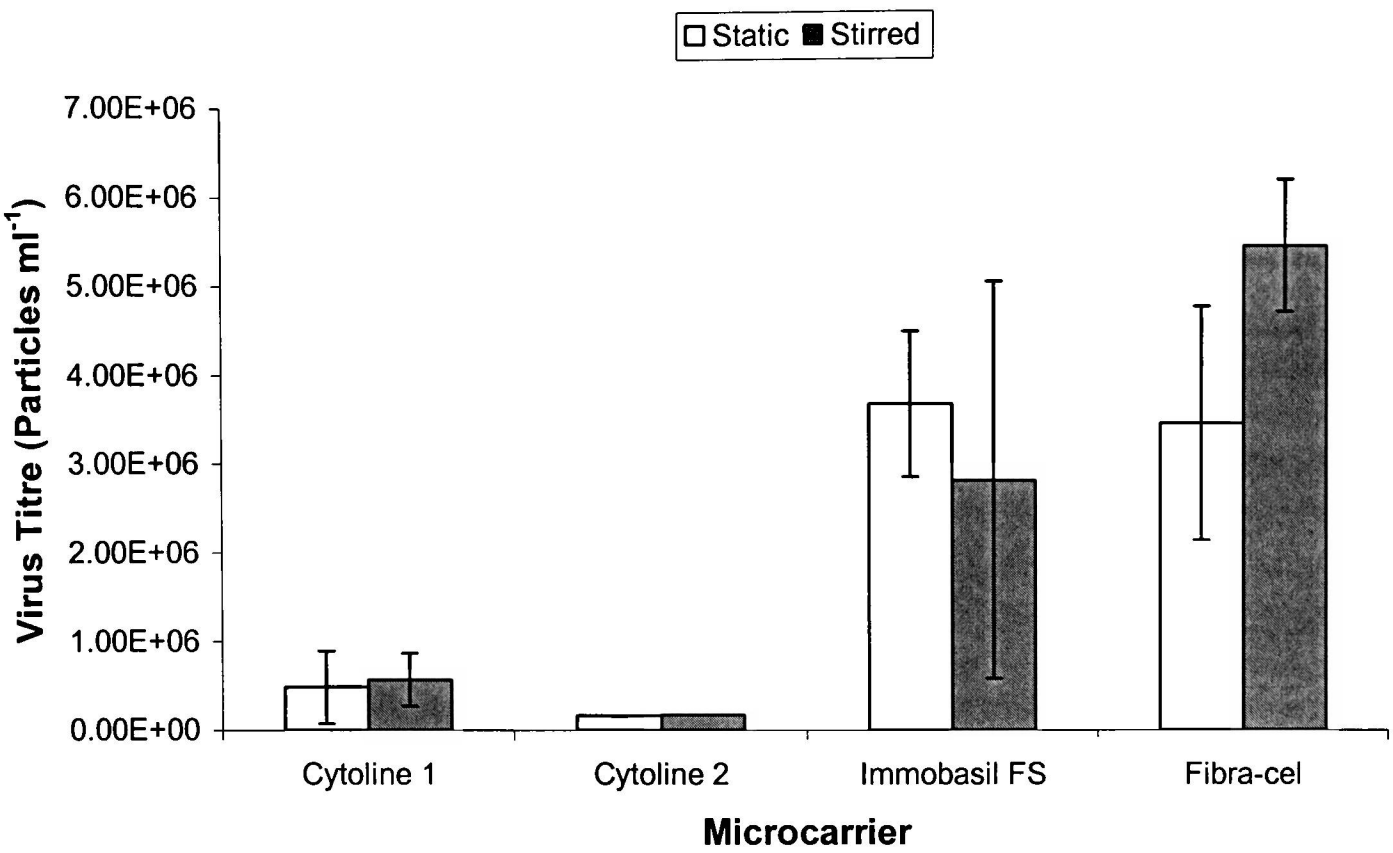


Figure 3.3.5.7

Mean final virus titres a batch culture of TEFLYRD/83 cells in 100ml spinner flasks (20ml working volume) using different microcarriers. Cells were attached to carriers (2ml carrier volume) and cultivated for 4 days under either static or stirred conditions (40rpm). Supernatant was harvested on the 5^{th} day and the virus titre was measured using RT-PCR. Error bars represent observed range, n = 2.

3.4 Conclusions

In this chapter the fundamental characteristics of the TEFLYRD/83 cell line have been studied to facilitate the optimisation of viral vector production. Monolayer cultures were carried out to determine the optimal mode of operation. From these experiments it is apparent that semi-continuous cultures offer the ability to produce infectious virus particles over long periods of time. The total virus production is less than that observed in batch cultures; however, a reduction in temperature to 32°C during virus production resulted in far superior titres being obtained. Therefore, the ideal mode of operation for alternative culture systems would be a semi-continuous culture with a cell growth phase at 37°C, followed by a production phase at 32°C, once cells had reached maximum density.

Porous microcarriers offer great potential for high density, long term cultivation of anchorage dependent cells. It has been shown that TEFLYRD/83 cells rapidly attach to various types of carrier and can be successfully cultivated in static and stirred cultures. Virus production is enhanced by agitation within the vessel, as this allows for effective transport between the internal and external environments. Of the four microcarriers that were tested, Fibra-Cel™ offered the greatest potential with regard to virus titre and specific cell productivity. However, this microcarrier may not be suitable for all applications due to its geometry. In the case of stirred tank and fluidised bed reactors, Cytoline 1 microcarriers should be used. In these experiments, this carrier supported rapid cell growth, which became limited after 3 days. This unfortunately led to a sub-optimal final virus titre. It is thought that this microcarrier is capable of sustaining high virus productivity under the correct culture conditions.

Chapter 4: Alternative Bioreactor Systems for Retroviral Vector Production

4.1 Introduction

Recombinant retroviruses are currently used in the majority of gene therapy trials (Kang *et al.* 2000; McTaggart and Al Rubeai 2001;Wu and Ataai 2000; Wu *et al.* 2002). The advantages of these vectors are that they can be easily manipulated in vitro, the vector integrates stably into the host chromosome without any apparent pathogenicity, a wide range of target cells can be infected, and reasonably large genes can be inserted into the vector (Kim *et al.* 2000). However, the disadvantages of this system are that target cells must be actively dividing for vectors to enter them (Smith 1995) and their titres are relatively low, usually in the region of 10^7 particles ml^{-1} for roller bottle cultures. Another limiting factor is the short half life, which is in the range of 2-8 hours at 37°C (Higashikawa and Chang 2001; Kaptein *et al.* 1997; Le Doux *et al.* 1999; Merten *et al.* 2001). For these reasons it is necessary to have a system capable of supporting a high cell density and a high specific productivity. Cell immobilisation within macroporous microcarriers has proved to be an effective method for cultivation of anchorage dependent and suspension cell lines, and of intensifying production of a number of biologics. In particular, packed bed bioreactors have shown great promise for the production of retroviral vectors (Kang *et al.* 2000; Merten *et al.* 2001). It has also been shown that virus titres can be greatly improved by reducing the culture temperature to 32°C (Cruz *et al.* 2000; McTaggart and Al Rubeai 2000). The reduction in temperature influences virus titre in two ways; firstly it is able to influence the specific production rate, although this is a weak function of temperature. Secondly, virus particles are more stable at lower temperatures so they

will not decay as rapidly at 32°C as they do at 37°C, enabling higher titres to be attained (Le Doux *et al.* 1999).

In this chapter, the potential of three alternative culture systems have been compared with roller bottles; the spinner basket packed bed bioreactor, a packed bed bioreactor with internal silicone tubing to allow efficient oxygen transfer within the bed, and a microcarrier culture in spinner flasks. To overcome the problem of the short vector half-life a two-stage culture method was used. The first stage is a cell propagation phase where the culture temperature is 37°C. Once cells were considered to be confluent, based on glucose consumption, the temperature was reduced to 32°C for the production of virus particles.

Alternatively, high virus titres may be attainable by using perfusion culture. Cells are retained in the culture vessel at 37°C while active virus particles are constantly removed and transferred to a harvest vessel at a lower temperature to reduce virus decay. This has the advantage that cells will remain at their optimal temperature and metabolism will not be hindered, leading to a higher production rate, and virus particles will not be affected by medium components that may be present at 32°C, such as proteoglycans, and have an adverse affect on their transduction efficiency. Therefore, four systems were tested in perfusion mode; a stirred tank bioreactor, a packed bed bioreactor with internal silicone tubing, a fixed bed bioreactor, and a fluidised bed bioreactor.

4.2 Materials & Methods

4.2.1 Semi-continuous culture at 32°C

In the previous chapter it was concluded that the most efficient mode of producing infectious virus particles was semi-continuous culture, with a cell growth phase at 37°C and a production phase at 32°C. This method was used to compare three

scaleable systems to roller bottle culture and assess their potential for large-scale production. The systems that were evaluated were microcarrier culture in spinner flask, a packed bed bioreactor, and a spinner basket culture.

Roller Bottle Culture

Cells were harvested from T-150 culture flasks for seeding into roller bottles (Falcon). Each roller bottle was inoculated with a total of 6.8 x 10^7 cells in 150ml of pre-warmed culture medium. The bottles were incubated at 37°C on a roller bottle rack, running at 0.25 rpm. After 24 hours the roller bottle rotation speed was increased to 0.5 rpm. The cells were incubated for a total of 3 days at 37°C to allow them to grow to confluence. On the fourth day a complete medium change was performed and the volume was reduced to 100ml. The temperature was reduced to 32°C. The cells were incubated for 2 days to acclimatise to this temperature. A complete medium change was performed on the fifth day and the volume was reduced further to 50ml. A daily medium change was performed for the following 10 days. Virus-containing culture supernatant was harvested daily and stored in liquid nitrogen for later measurement of the virus titre by RT-PCR. The glucose concentration in the supernatant was measured daily in order to monitor cell viability and growth. At the end of the experiment cells were washed with PBS and detached from the roller bottles using trypsin, and counted using a heamocytometer under a microscope to determine the final cell number.

Microcarrier culture in 100ml Spinner Flasks

The producer cells were cultured on 10ml Immobasil FS microcarriers (Ashby Scientific, UK) in 100ml spinner flasks (Bellco™, USA) with 90ml of culture medium. Microcarriers were prepared as described in 2. The flasks were inoculated with 50ml of culture medium containing 2 x 10^6 cells ml^{-1} of microcarrier of

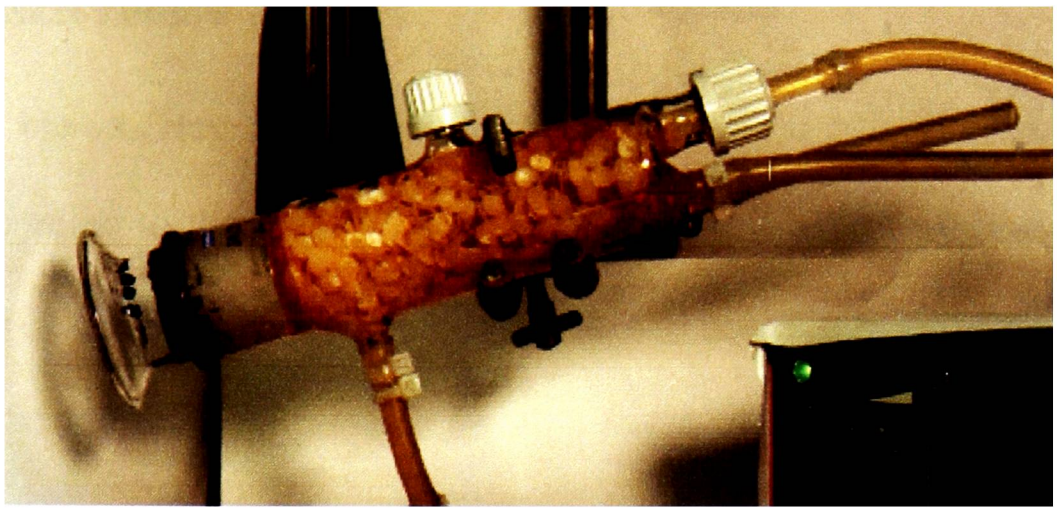
exponentially growing cells. The flasks were placed in an incubator during the attachment phase and gently shaken every 30 minutes to obtain uniform distribution of cells on the carriers. A sample of supernatant was taken every hour and the number of cells in suspension was counted under a microscope, using a haemocytometer, to estimate the number of attached cells. After 4 hours, culture media was added to give a total final volume of 100ml. A sample of microcarriers was taken and the cell density was estimated using MTT (see chapter 2) to ensure that the disappearance of cells from the supernatant was due to attachment to the carriers. The flasks remained under static conditions overnight in a humidified incubator with 5% CO_2 at 37°C. Duplicate flasks were stirred at 40 rpm using a magnetic stirrer. Samples of microcarriers were taken at regular intervals and the cell density was estimated using MTT. The residual glucose concentration was measured daily and medium was changed when required to maintain the glucose concentration above 1g/L. The cultures were incubated at 37°C until the cells reached the stationary phase. A complete medium change was performed and the temperature was lowered to 32°C. Cells were incubated for 2 days to acclimatise. A daily medium change was performed for the following 10 days. Virus-containing culture supernatant was harvested at regular intervals and stored in liquid nitrogen for later measurement of the virus titre by RT-PCR.

Packed bed bioreactor

The packed bed bioreactor (PBR) was filled with 5g of Fibra-Cel™ discs (Bibby Sterilin, UK). The vessel was assembled as a closed system with a 250ml Duran bottle (Figure 4.2.1.1). The discs were prepared by circulating PBS through the bed, using a peristaltic pump, to hydrate them for several hours before the complete system was autoclaved at 121°C, 1 bar pressure for 30 minutes. Under sterile conditions the PBS

was completely removed and replaced with 200ml of culture medium. The vessel was placed in an incubator at 37°C and air + 5% CO_2 gas was used to aerate the bed. The medium was circulated through the packed bed for a minimum of 24 hours to equilibrate the Fibra-Cel™ discs. The PBR was inoculated with 2.5 x 10^7 cells via the top port. Cells attached within 2 hours, after which medium re-circulation was commenced. The vessel was incubated at 37°C and aerated with air + 5% CO_2. Glucose was measured on a regular basis and the medium was changed every few days in order to maintain the glucose concentration >1g/L. Once the cells had reached steady state a complete medium change was performed and the temperature was reduced to 32°C. The cells were incubated for two days to acclimatise before the production phase was started. During retrovirus production medium was changed daily over a period of 10 days, and the supernatant was stored in liquid nitrogen before measuring the virus titre by RT-PCR. The cell number was determined at the end of the culture by using an MTT assay.

A



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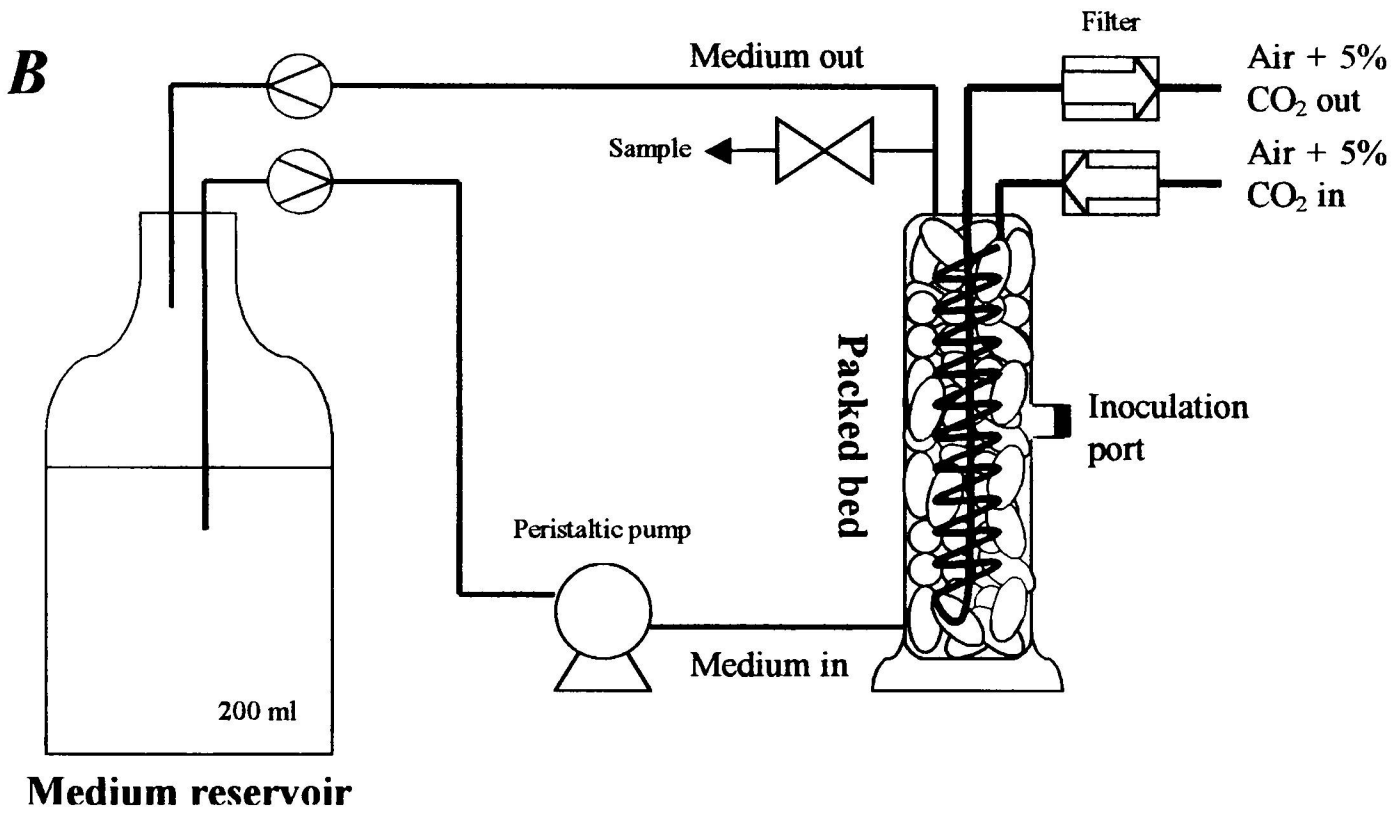


Figure 4.2.1.1

Photograph of the packed bed bioreactor system (A) and schematic of the experimental set-up (B). Medium contained in the reservoir bottle was circulated through the packed bed by means of a peristaltic pump. An internal silicone tube was used to supply air + 5% CO2 to cells immobilised within 5g (50ml) Fibra-Cel ™ discs in the packed bed.

Spinner basket culture

The spinner basket (New Brunswick Scientific, USA) was assembled for bubble-free aeration according to the manufacturer's instructions, with 10g (equivalent to 100ml bed volume) of Fibra-Cel™ discs packed in the basket (Figure 4.2.1.2). Prior to autoclaving at 121°C, 1 bar pressure for 30 minutes, 400ml of PBS was added to the vessel. All following procedures were performed within a laminar flow cabinet to maintain sterility. The PBS was removed and replaced with 400ml of culture medium to equilibrate the carriers. The spinner basket was incubated overnight at 37°C and aerated with air + 5% CO_2. The total cell number required for inoculation was 5×10^7 cells. After removing the equilibration medium the cell suspension was added to the vessel and fresh medium was added as required to give a total working volume of 500ml. The flask was placed on a magnetic stirrer within an incubator at 37°C and aerated with air + 5% CO_2. Residual glucose was monitored and the medium was changed as necessary to ensure the concentration was >1g L^{-1}. Once the cells had reached steady state (typically 7-8 days) a complete medium change was performed and the temperature was reduced to 32°C. The cells were acclimatised for two days before the production phase was started. During the production phase, medium was changed daily for a period of 10 days, and the supernatant was stored in liquid nitrogen before quantifying the virus titre with RT-PCR. Spent medium was also analysed for glucose consumption and lactate production. The cell number was determined at the end of the experiment by using an MTT assay.

A

B

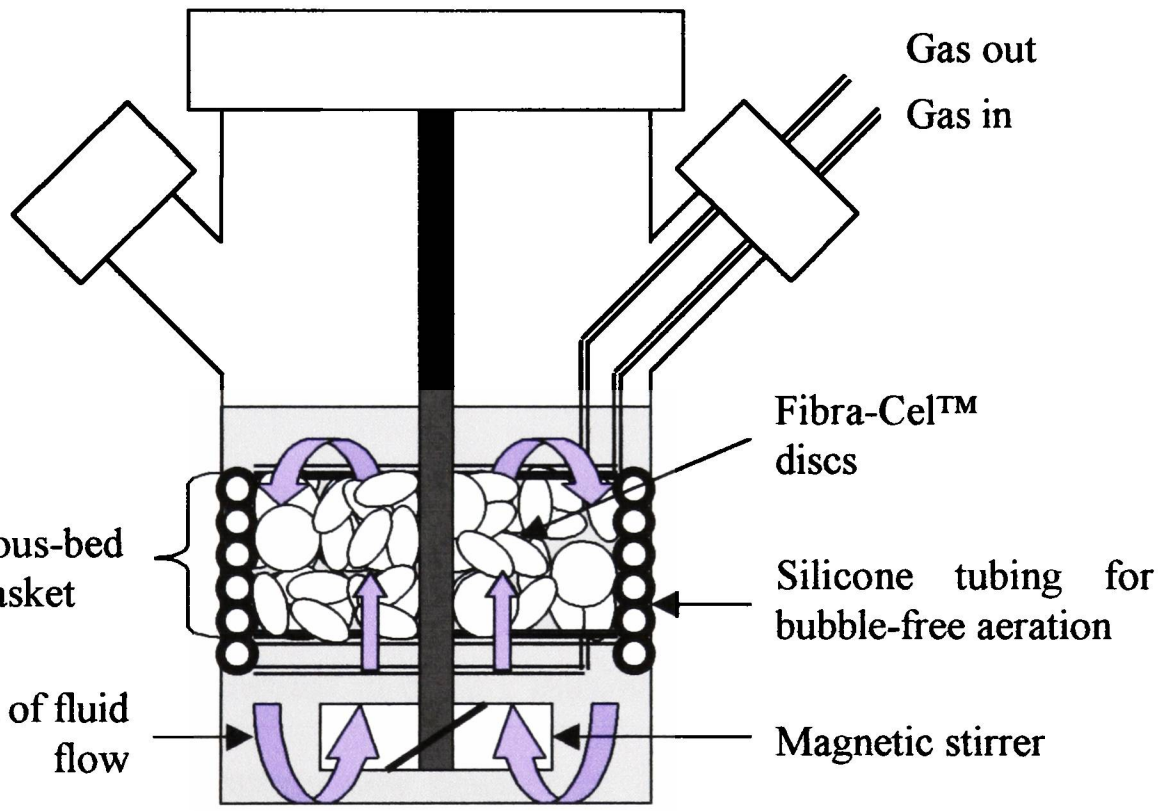


Figure 4.2.1.2

Photograph of the spinner basket (A) and schematic of the experimental set-up (B). Cells were immobilised on Fibra-Cel™ discs within the basket. Virus particles were secreted into the culture medium as it was circulated through the bed by an up-pumping impeller.

4.2.2 Comparison of Perfusion Culture Systems

An alternative method, and possibly a more effective approach for producing high titre virus stocks, might be to develop perfusion culture systems that rapidly remove newly formed virus particles from the high temperature cell culture vessel, where particles rapidly lose their activity, to a low-temperature vessel where viruses are more stable (Le Doux *et al.* 1999).

Stirred Tank Bioreactor Perfusion Culture

Cytoline 1 microcarriers were prepared as previously described and 100ml were added to a 1 litre (working volume) stirred tank bioreactor (LSL Biolafitte, France) with 900ml of PBS. The complete system (see Figure 4.2.2.1) was autoclaved at 121°C, 1 bar for 30 minutes. Following sterilisation the PBS was drained into the waste bottle and 900ml of culture medium was added to the vessel to equilibrate the microcarriers for a minimum of 24 hours. The culture set points were: temperature 37°C; DO_2 50%; pH 7.0; agitation speed 100rpm.

Prior to inoculation the medium was removed from the vessel. The vessel was inoculated with 1-2 x 10^6 cells ml^{-1} microcarrier. Cells were prepared in T-flask cultures. When the required numbers of cells were available, the cells were detached by trypsin digestion and suspended in culture medium. Medium was added to give a final volume of 1 L. The culture was agitated at 100rpm for 3 minutes every 30 minutes for 4 hours to allow uniform distribution of cells on the microcarriers. The system was left under static conditions for 24 hours before stirring was commenced at 100rpm.

Samples were taken at regular intervals and the glucose was measured to check cell viability. The perfusion rate was adjusted to maintain the glucose concentration above

1 g L^{-1} in the bioreactor. Samples of the supernatant were stored in liquid nitrogen and the virus titre was measured at a later time using the LacZ titration assay.

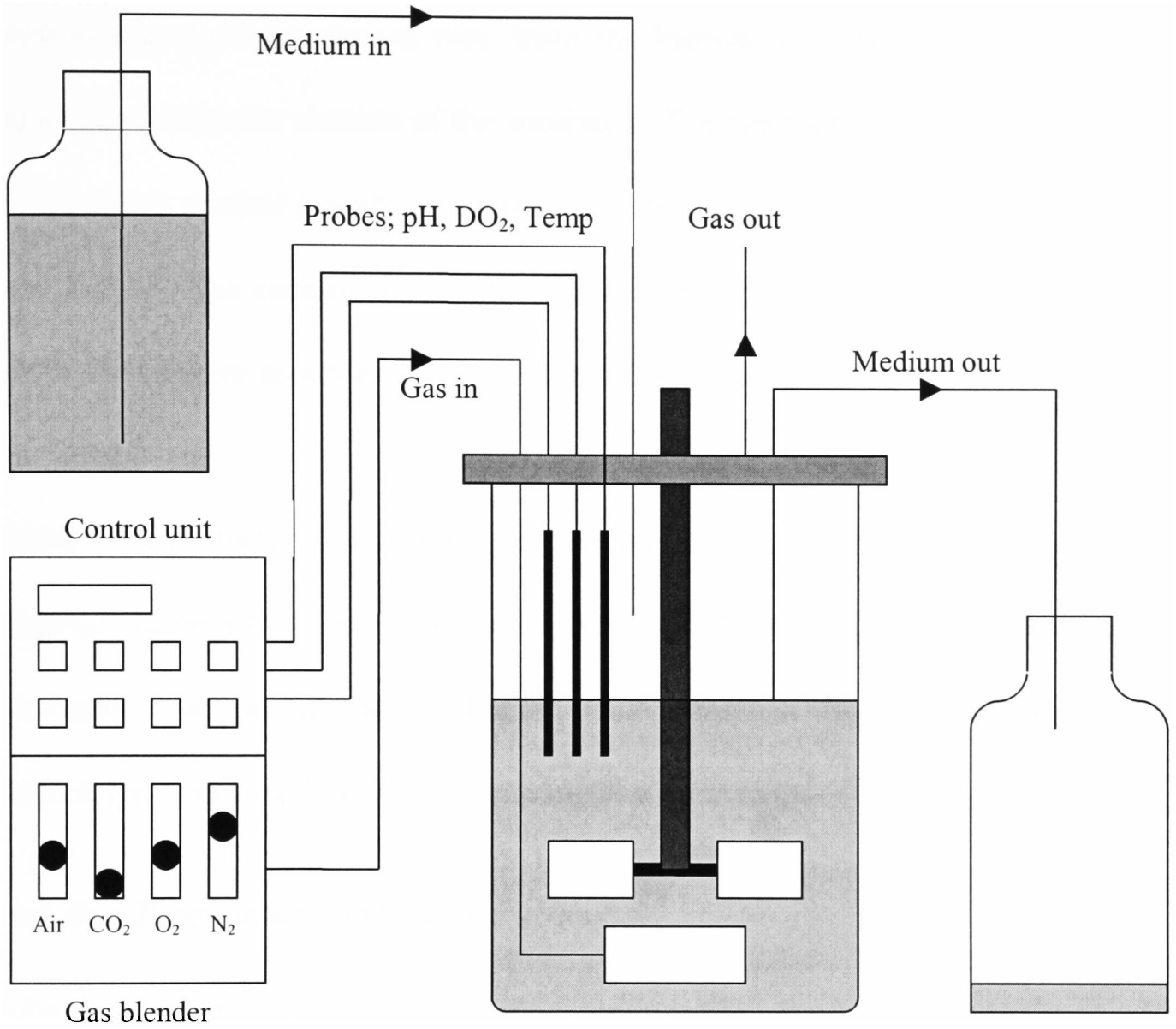


Figure 4.2.2.1

Schematic diagram of the experimental set up of the stirred tank perfusion culture. 100ml of Cytoline 1 microcarriers were suspended on 900ml of culture medium. The stirred tank was fitted with a six-bladed Rushton turbine. The culture set pints were: temperature 37°C; pH 7.0; DO_2 50%; agitation speed 100rpm.

Packed Bed Bioreactor Open Perfusion Culture

The packed bed bioreactor was prepared as described in section 4.2.1 with the exception that the downstream tube from the bioreactor vessel was connected to a separate harvest bottle outside of the incubator. The medium reservoir was connected to a bioreactor control system to control the upstream culture conditions as shown in Figure 4.2.2.2. The culture set points were as follows: temperature 37°C; DO_2 75%; pH 7.0. These were assumed to stay constant between the medium reservoir and the vessel inlet.

Samples were collected downstream of the packed bed and the glucose was measured at regular intervals to monitor cell growth and determine the perfusion rate. Supernatant was periodically collected and stored in liquid nitrogen for virus measurement using the LacZ titration assay at a later stage.

Fixed Bed Bioreactor Perfusion Culture

The fixed bed bioreactor system (BioEngineering, Germany) consists of the fixed bed compartment, containing 1.5g Fibra-Cel™ discs, within a vessel for conditioning the medium (see Figure 4.2.2.3). Following sterilisation at 121°C at 1 bar for 30 minutes, culture medium was added to the vessel and recirculated through the fixed bed to allow the discs to equilibrate for at least 24 hours. This medium was drained and the vessel was inoculated with 2×10^5 cells ml^{-1} medium of exponentially growing cells. Medium recirculation was initiated after 3 hours at a rate of 1ml min^{-1}. This was gradually increased to 5 ml min^{-1} over the next 5 days.

The medium in the conditioning vessel was sampled at regular intervals and the residual glucose concentration was measured. Samples of the supernatant were stored in liquid nitrogen and the virus titre was measured at a later time using the LacZ

titration assay. The perfusion rate was set according to the demand of the cells and glucose was maintained above 1g l^{-1}.

Fluidised Bed Bioreactor Perfusion Culture

Prior to the start of the experiment, 300 ml of Cytoline 1 microcarriers (Amersham Pharmacia Biotech) were prepared as described in chapter 2. The carriers were transferred to the fluidised bed bioreactor (Cytopilot mini; Amersham Pharmacia Biotech) and 1 litre of distilled water was added. The complete system was sterilised for 30 minutes at 121°C, 1 bar pressure in an autoclave. Using sterile technique, the medium tank and the harvest vessel were connected to the fluidised bed bioreactor. The water was removed from the bioreactor via the sampling port and replaced with 2 litres of pre-warmed culture medium. The pH was adjusted to 7.0 and the dO_2 to 40-50% saturation. The microcarriers were left overnight to equilibrate.

The culture was inoculated with 2 x 10^6 cells ml^{-1} microcarrier. Cells were prepared in T-flask cultures. When the required numbers of cells were available, the cells were detached by trypsin digestion and suspended in culture medium. Cells were required to be in the exponential phase. The fluidised bed bioreactor was drained of the equilibrium medium and the inoculum bottle was connected to transfer the cells to the vessel. The bioreactor was filled to the working volume (2 litres) with fresh culture medium. To enable a uniform distribution of cells on the carriers, the fluidised bed bioreactor was run in packed bed mode at 100 rpm with fluidisation of the bed for 10 seconds every 30 minutes at 150 rpm. This was repeated for 3 hours. After 3 hours a sample of supernatant was taken and the cells in suspension were counted using trypan blue exclusion test and a heamocytometer under a microscope. This was used to determine the number of cells that had attached to the carriers. The carriers were

fluidised at 350rpm to give 100% fluidisation. The perfusion rate was adjusted to maintain the glucose concentration above 1.5g L^{-1}.

Samples were taken at regular intervals and the glucose concentration was measured to determine the perfusion rate. Cell growth was monitored by calculation of the glucose uptake rate. Samples of the supernatant were stored in at -80°C before virus titration using the LacZ titration assay.

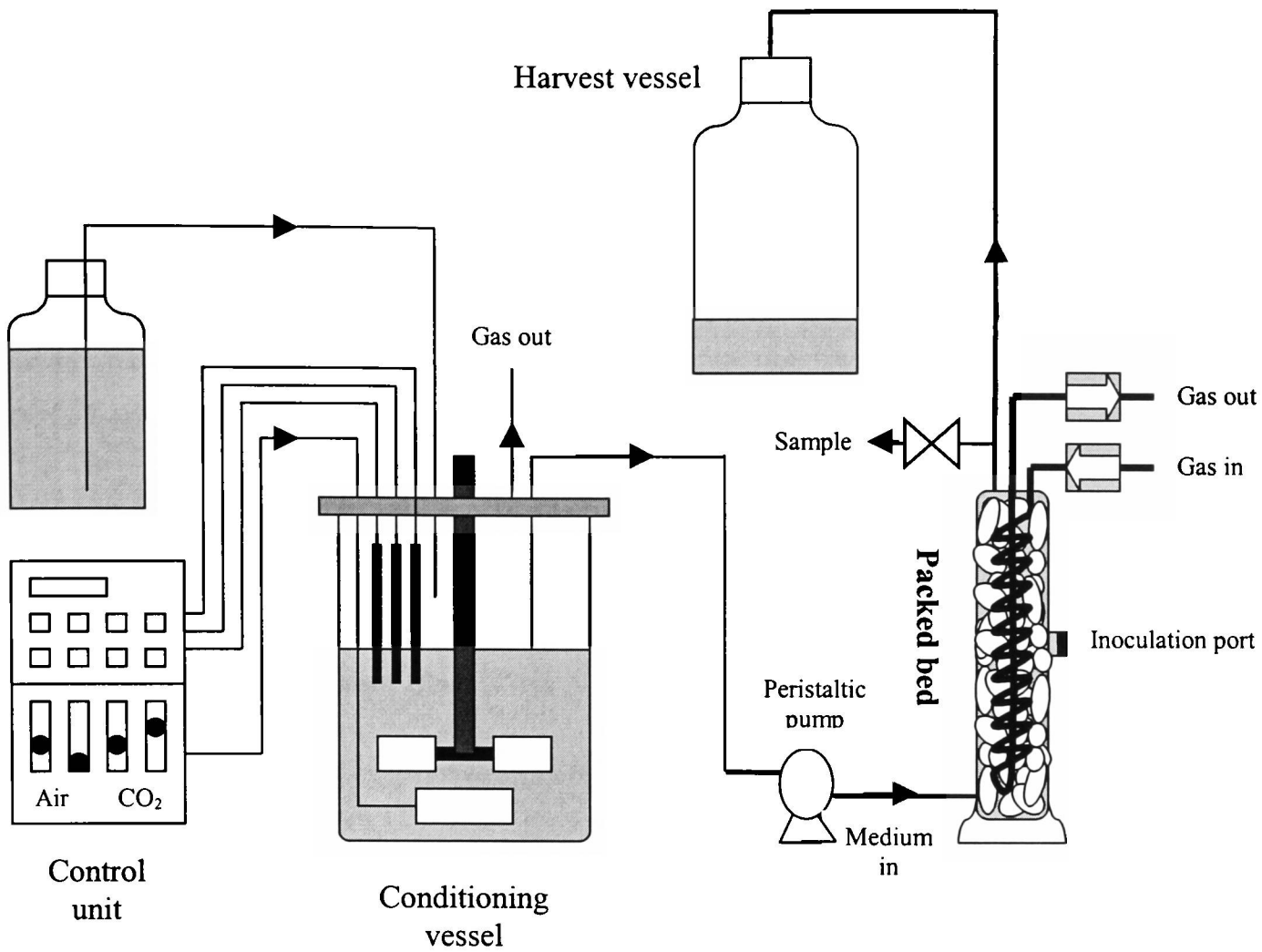


Figure 4.2.2.2

Schematic diagram of the experimental set-up of the open perfusion packed bed culture system. The packed bed was filled with 5g Fibra-Cel™ discs and placed in a 37°C incubator. The culture set points for the conditioning vessel were: temperature 27°C; pH 7.0; DO_2 75%.

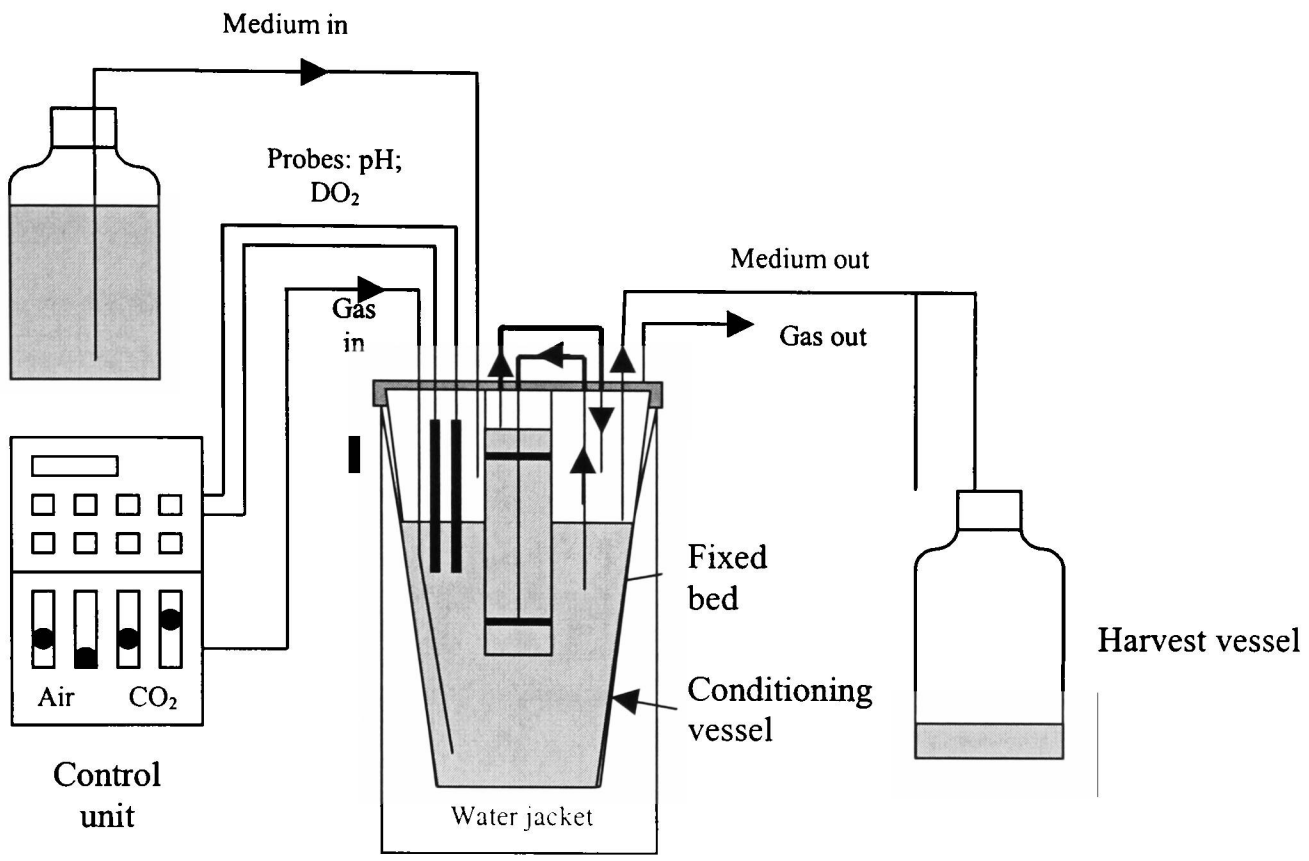


Figure 4.2.2.3

Schematic diagram of the experimental set up of the fixed bed perfusion bioreactor. The fixed bed was filled with 15ml of Fibra-Cel™ discs. Medium was circulated between the conditioning vessel and the fixed bed at a maximum flow rate of 5 ml min^{-1}. The set points were: temperature 37°C; DO_2 50%, pH 7.0. The temperature of the vessel was control by circulating water through an external jacket from a water bath.

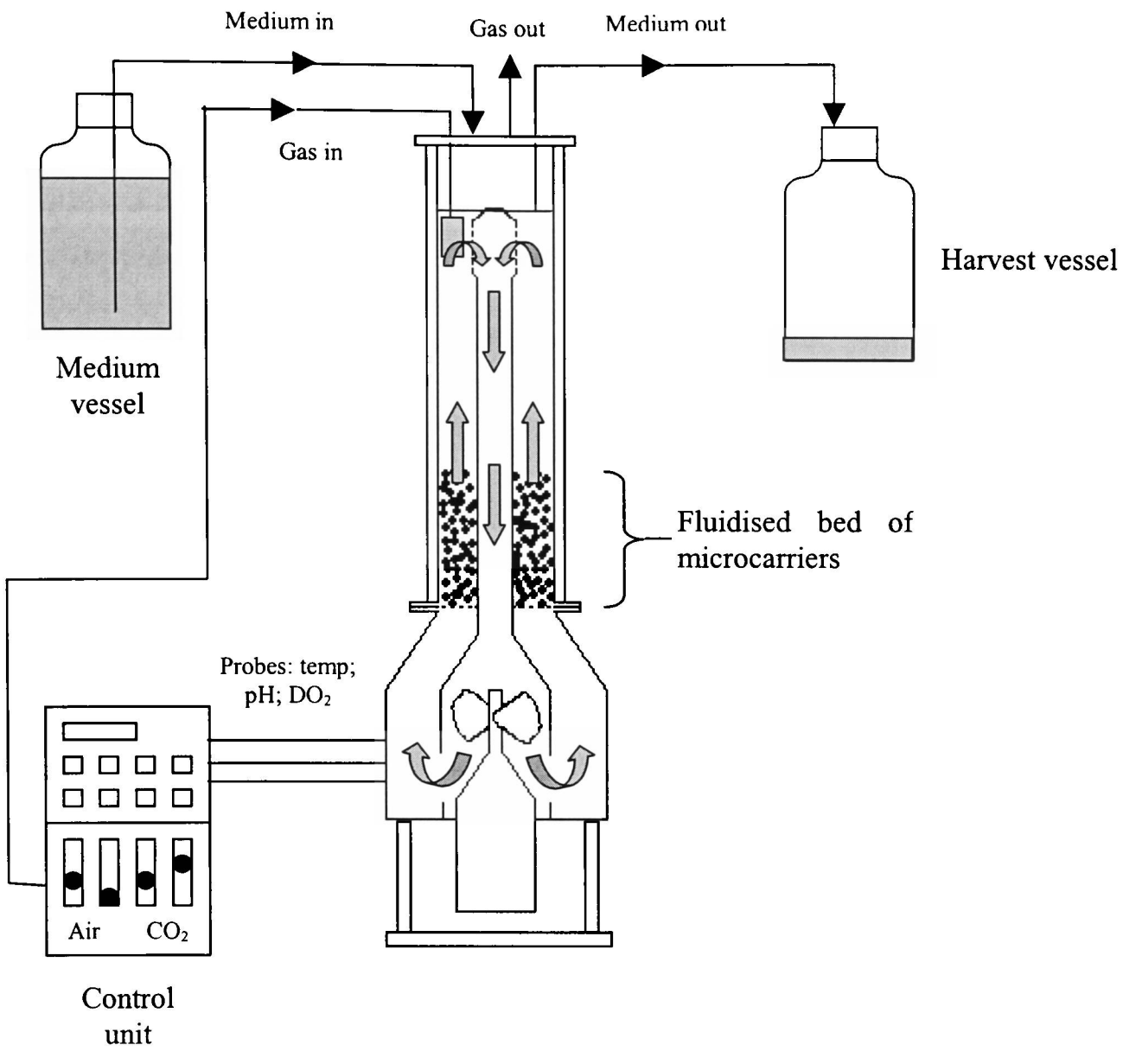


Figure 4.2.2.4

The schematic diagram of the experimental set up of the fluidised bed bioreactor. Medium was circulated through the bed by a magnetically driven axial flow impeller. The culture set points were: temperature 37°C; pH 7.0; DO_2 50%; impeller speed 350rpm.

4.3 Results and Discussion

4.3.1 Comparison of semi-continuous culture systems at 32°C

The three systems tested, the spinner flask with Immobasil FS microcarriers, the spinner basket from New Brunswick Scientific and the packed bed bioreactor (PBR) with Fibra-Cel™ discs, were compared to roller bottle production. From these it could be clearly shown that the TEFLYRD producer cells responded differently to the environmental conditions in each system. In the PBR and the Spinner basket, the ratio of medium:discs was kept the same. The performance of these reactors was evaluated with regard to final cell density, maximum virus titre, daily vector production, and average specific production rate. As it was not possible to determine the cell number in immobilised cultures directly, the cell growth and viability during the experiment was determined by the Glucose Uptake Rate (GUR). The GUR (g day^{-1}) was calculated as follows:

$$GUR = \frac{([S]_0 - [S]_t) \times V}{(t - t_0)}$$

where $[S]_0$ and $[S]_t$ are the glucose concentration (g l^{-1}) at time, 0 and time, t, respectively, and V is the medium volume (litres). From this it was possible to estimate the specific production rate in each system. Each system was run as a semi-continuous culture with a complete medium change performed every day during the production phase. The culture parameters are summarised in Table 4.3.1.1.

Table 4.3.1.1

Cell growth and retrovirus production parameters for semi-continuous cultures at 32°C in various culture systems.

	Roller bottle	**Spinner basket**	**Packed bed Bioreactor**	**Spinner flask**
Type of carrier	None	Fibra-Cel™	Fibra-Cel™	Immobasil-FS
Initial cell number (x 10^6 cells)	68	50	25	20
Final cell number (x 10^6 cells)	239	680	550	41.1
Medium volume (ml)	50	400	200	90
Carrier volume (ml)	N/A	100	50	10
Maximum virus titre (LTU ml^{-1} day^{-1})	1.95x 10^7	6.92 x 10^6	1.21 x 10^7	5.25 x 10^6
Average daily virus production rate (LTU ml^{-1} day^{-1})	**1.48 x 10^7**	**3.44 x 10^6**	**9.24 x 10^6**	**4.56 x 10^6**
Total virus production	**7.4 x 10^9**	**1.38 x 10^{10}**	**1.8 x 10^{10}**	**4.56 x 10^9**
Specific virus production rate (LTU $cell^{-1}$ day^{-1})	4.09	0.778	3.23	0.867

4.3.2 Cell growth and metabolic trends

Roller bottle culture is currently the preferred method for the production of clinical grade retrovirus particles. From these experiments, it was possible to achieve a final cell density of 4.8 x 10^6 cells ml^{-1}. For 850cm^2 roller bottles with 50ml of culture medium, this is equivalent to 2.8 x 10^5 cell cm^{-2}. This density is consistent with monolayer cultures in T-flasks. During the production phase the glucose uptake rate was calculated as being 0.167 – 0.183 g day^{-1} (see Figure 4.3.2.1) and the residual glucose concentration after 24 hours was ~0 mM. This demonstrates that glucose was the limiting factor, not surface area, as can be seen from Figure 4.3.2.2, and that the cell number achieved was the maximum that could be supported by 50ml of culture medium.

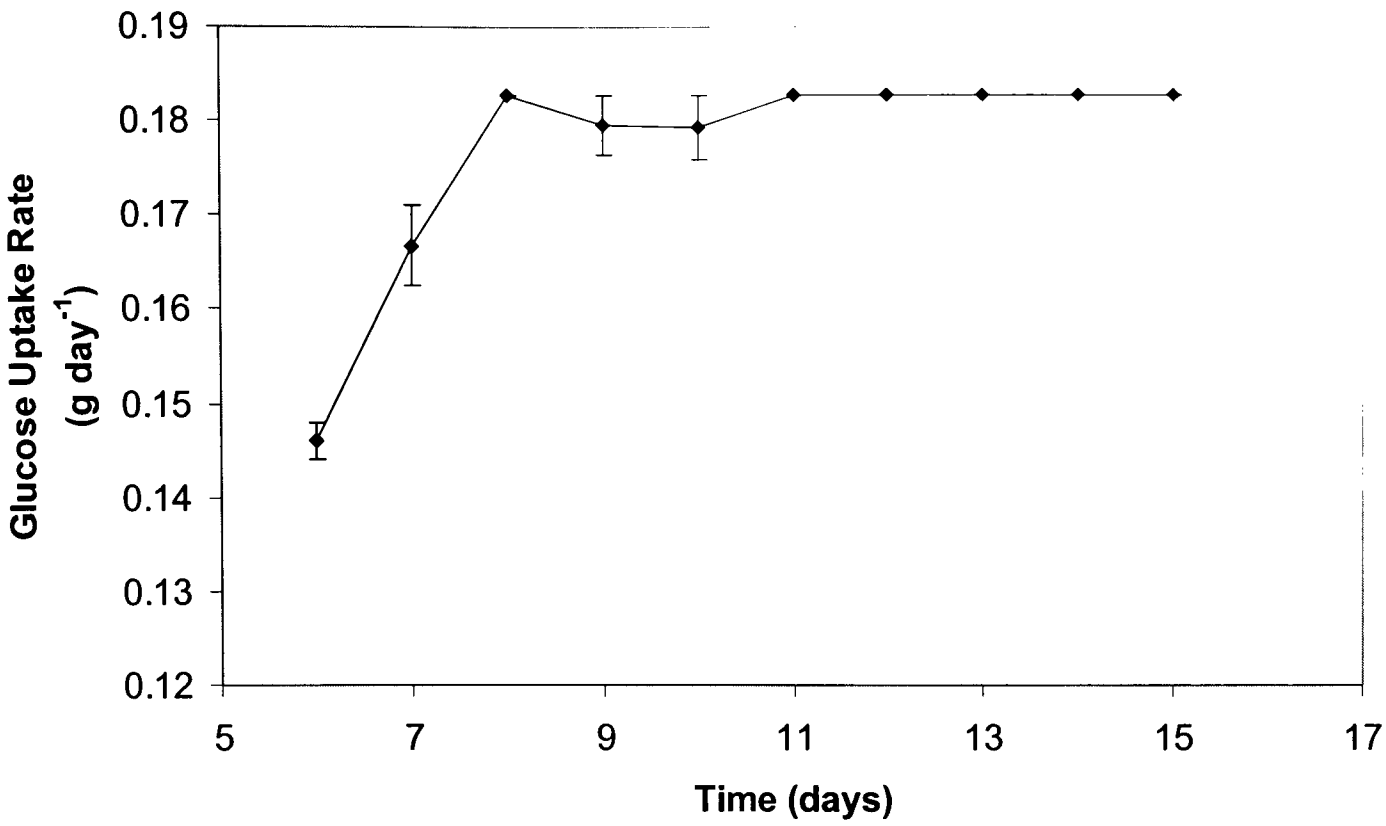


Figure 4.3.2.1

Mean glucose uptake rate (GUR) for TEFLYRD/83 cells in roller bottle culture. Cells were cultured at 37°C for the first 3 days before the temperature was reduced to 32°C. The culture medium was changed daily from day 5 onwards. Error bars represent observed range, n = 2.

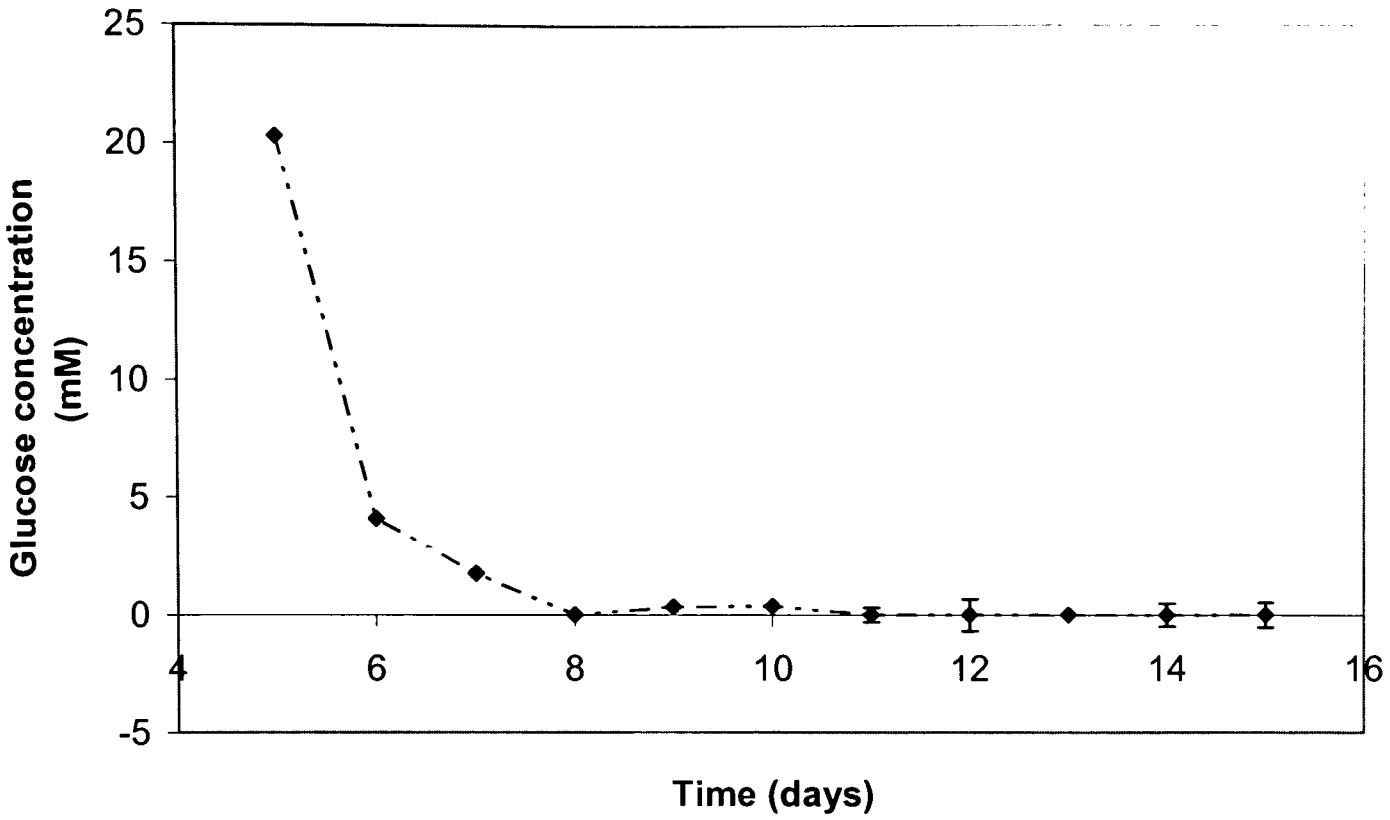


Figure 4.3.2.2

The residual glucose concentration for TEFLYRD/83 cells in roller bottle culture. A complete medium change was performed during the virus production phase (day 5 - 15) when the culture temperature was at 32°C. The figure shows that glucose was completely utilised by the cells and limiting from day 8 forwards. Error bars represent observed range, n = 2.

Of the three alternative systems that were tested, the highest final cell number was achieved in the spinner basket. This system was capable of supporting 6.8 x 10^8 cells. This is equivalent to a cell density of 6.8 x10^6 cells ml^{-1} carrier. However, the PBR produced 5.5 x 10^8 cells but its total bed volume was half that of the spinner basket. Therefore, the cell density in this system was 1.1 x 10^7 cells ml^{-1} carrier. In the spinner basket and PBR, an equal ratio between medium volume and bed volume was used. During the production phase the maximum GUR in these systems was 0.684g day^{-1} and 0.686g day^{-1}, respectively (see Figure 4.3.2.3 and Figure 4.3.2.5). The yield coefficient for these systems was calculated as follows:

$$Y_{x/s} = \frac{cell\ number}{GUR}$$

Based on the final cell density and the relative GUR, $Y_{x/s}$ was 10^9 cells g^{-1} and 8 x 10^8 cells g^{-1} for the spinner basket and the PBR, respectively. The GUR is determined from the change in glucose, which is considered to be the primary energy source for the culture, and is represented by ΔS. As glucose serves as both carbon and energy source its utilisation can be fully written as:

$$(\Delta S) = (\Delta S)_{assimilation} + (\Delta S)_{growth\ energy} + (\Delta S)_{maintenance\ energy}$$

While the change in substrate due to assimilation is relatively constant the overall yield coefficient is not. Cells that are in the exponential phase will consume glucose for assimilation and growth energy, whereas cells in the stationary phase will consume glucose for maintenance energy as growth rate is essentially zero (Bailey and Ollis 1986). It was assumed that during the production phase the cells had reached the stationary phase and glucose was therefore consumed for maintenance. The higher yield coefficient in the spinner basket implies that more maintenance energy was required to sustain cell viability. This is caused by an increase in environmental stress

to cells. In a bioreactor this is commonly due to deprivation of glucose, serum, glutamine, essential amino acids and exposure to toxic metabolites such as ammonia and lactate. It may also be a reaction to changes in pH or oxygen limitation. In more severe cases this will lead to the cell engaging in a biochemical pathway comprising of a protease cascade, which leads to the induction of a range of enzymes which are responsible for the death and destruction of the cell (Fassnacht *et al.* 1999). This form of programmed cell death is known as apoptosis and is a common cause of cell density restrictions in industrial processes.

A common characteristic of packed bed bioreactors is the limitation of either nutrient or oxygen transfer rate. Over the 24 hour period between medium changes, the amount of residual glucose in the spinner basket did not fall below 5.5mM as illustrated in Figure 4.3.2.4. Therefore, a limitation in the supply of nutrients was not believed to be the reason for the increased maintenance energy. The dominant mechanism for supply of oxygen to immobilised cells in packed beds is intraparticle convective flow (Park and Stephanopoulos 1993). The extent of this is controlled by (i) the bead size, which determines the fraction of the fluid flow rate passing through the particle pores and (ii) the medium flow rate through the packed bed. Hence, a linear relationship exists between the flow rate through the packed bed and the total number of viable cells in the packed bed. Fluid flow through the spinner basket packed bed was achieved with a magnetically driven down-pumping axial flow impeller, with a stirring speed of 80rpm. Thus, it can be concluded that the type of impeller and/or the agitation speed were the cause of the relatively low cell number. The parameters used were unable to provide an oxygen transfer rate capable of supporting cell densities higher than 6.8×10^6 cells ml^{-1}, which is only 35% of the density achievable according to the manufacturer's reports and other published data (Merten *et al.* 2001; Shi *et al.* 1992; Wu and Ataai 2000). The oxygen transfer rate

was also believed to be the cause of the increase in maintenance energy compared to the PBR. Hence, cell number could be significantly improved by modifications in mixing regime.

In contrast, the PBR was believed to offer a more conducive environment for cell growth, resulting in a lower level of required maintenance energy. Fluid flow through the packed bed was achieved using a peristaltic pump. Hence the flow rate was sufficiently high enough to provide good transfer rates of nutrients to the cells by convective flow. Efficient oxygenation was also achieved by the internal silicone tubing in the bed. This prevented the appearance of gradients in the bed and helped to maintain a homogenous environment. The limitation in cell number was attributed to complete glucose consumption. The residual glucose in the medium reservoir fell as low as 1.4mM between medium changes as can be seen in Figure 4.3.2.6. There are two ways in which this limitation could be overcome. The first is to increase the frequency of the medium changes as suggested for the spinner basket. The alternative is to increase the volume of the medium reservoir. This is an option that is not available for the spinner basket as the packed bed is contained within the medium reservoir. Alternatively, these systems could be operated as continuous perfusion cultures where the medium feed rate could be adjusted according to the GUR of the cell population.

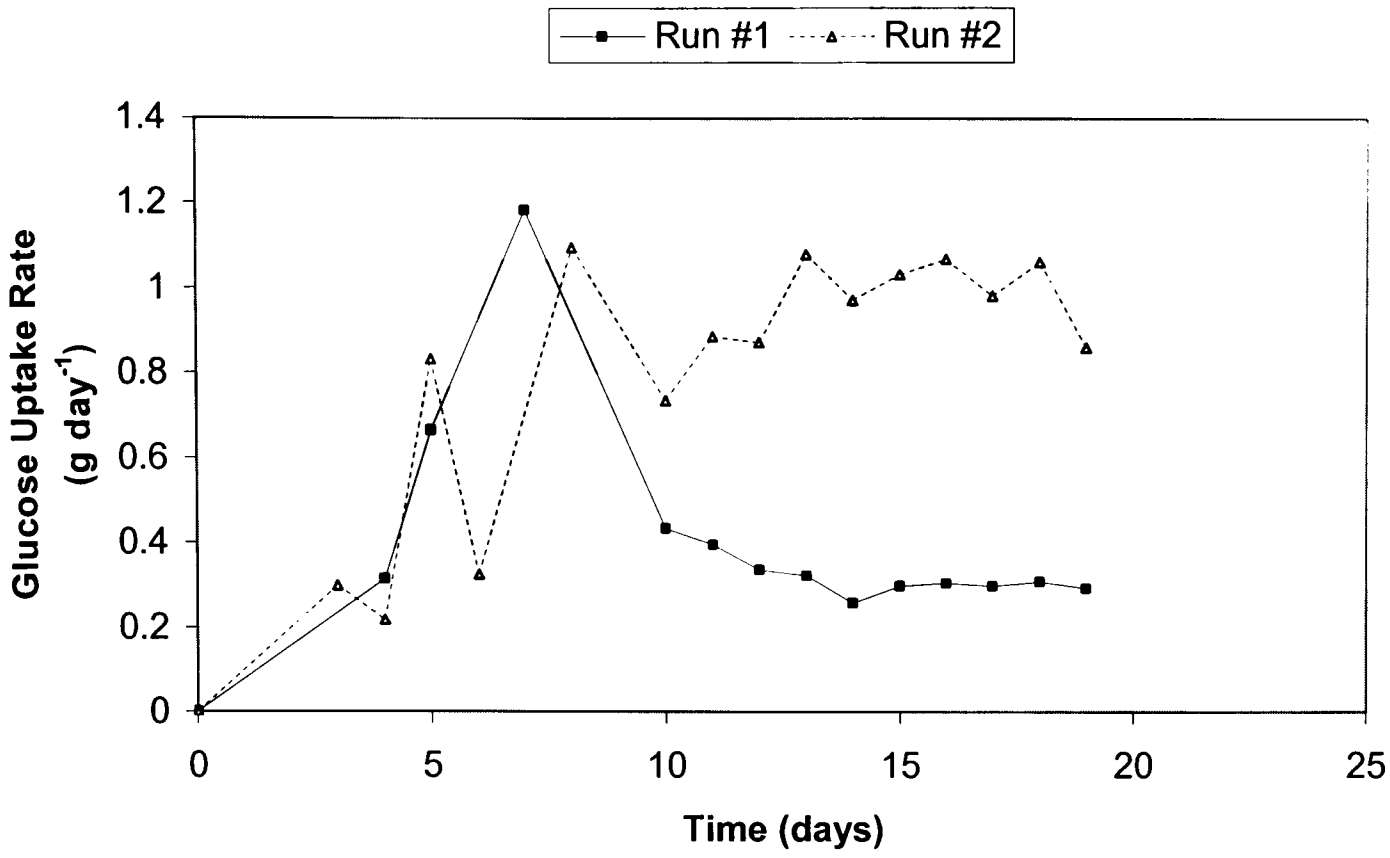


Figure 4.3.2.3

Glucose uptake rate (GUR) for TEFLYRD/83 cells in the Spinner basket packed bed bioreactor (New Brunswick Scientific). Cells were cultivated at 37°C for 7 days before the temperature was reduced to 32°C. Culture medium was changed daily thereafter and virus containing supernatant was stored in liquid nitrogen.

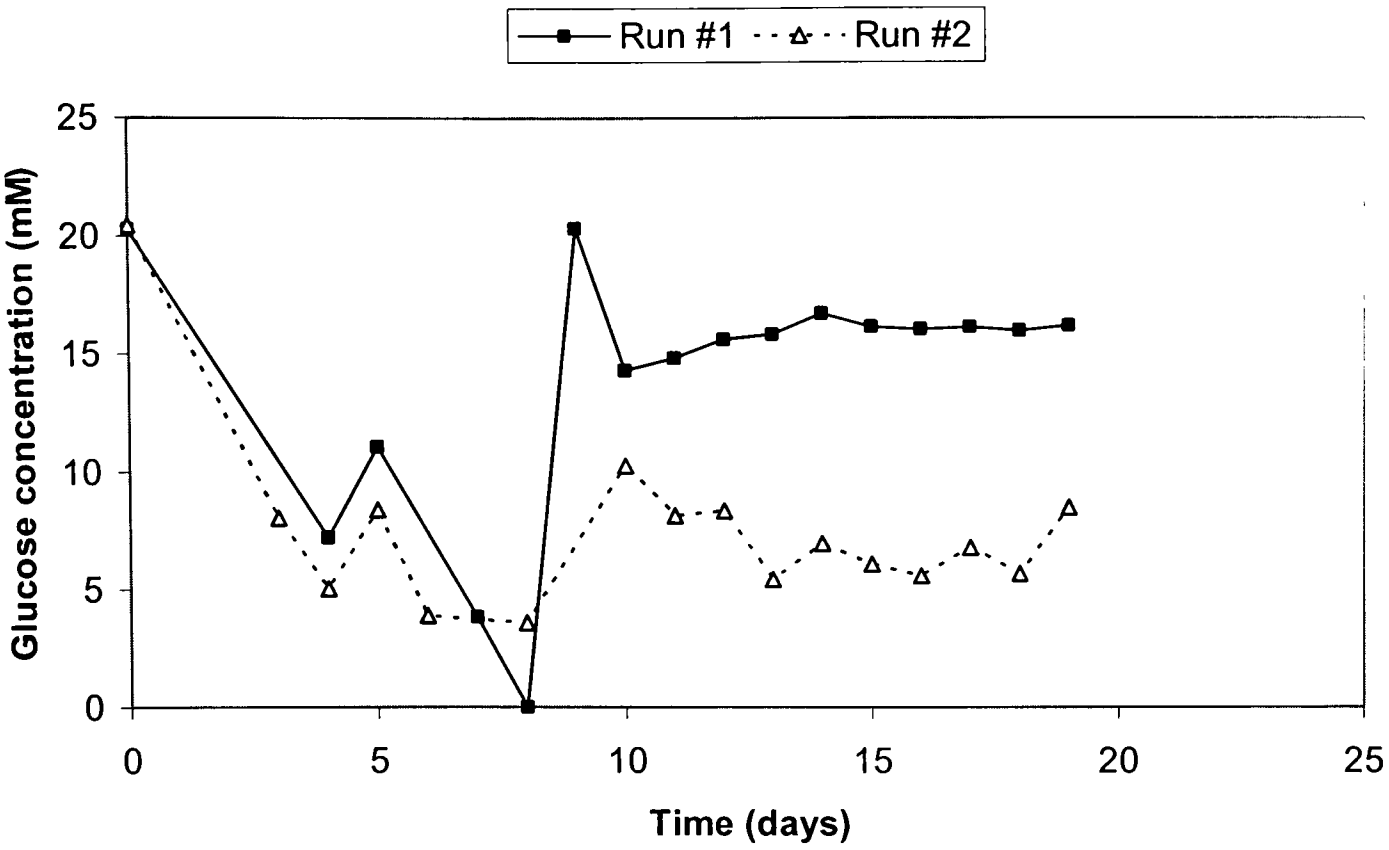


Figure 4.3.2.4

The residual glucose concentration in duplicate cultures of TEFLYRD/83 cells in spinner basket culture. Medium was changed after 4 days, 7days and daily after 9 days in order to prevent glucose from limiting cell growth.

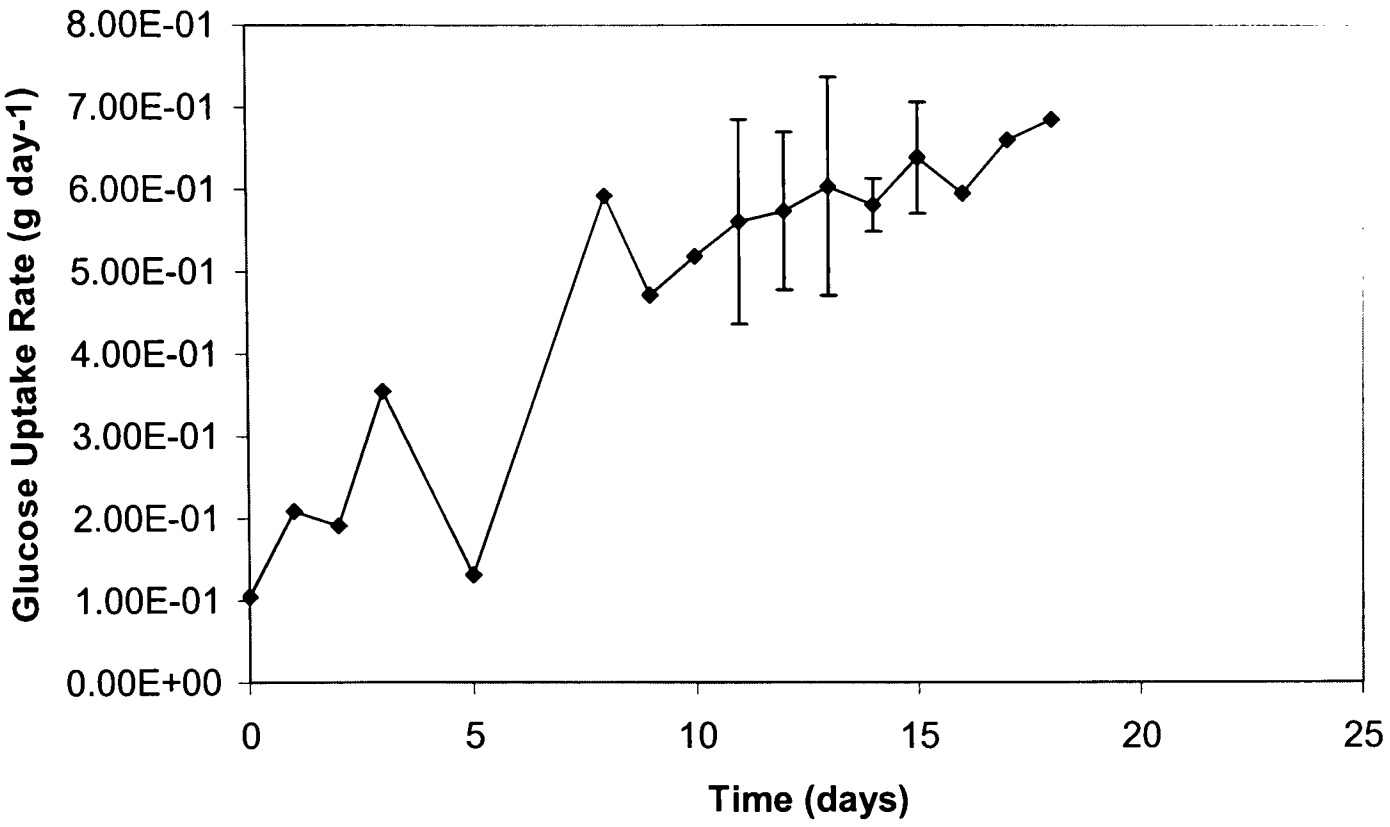


Figure 4.3.2.5

Mean glucose uptake rate (GUR) for TEFLYRD/83 cells in a packed bed bioreactor with internal aeration (see Figure 4.2.1.1). Cells were cultivated at 37°C for 7 days before the temperature was lowered to 32°C for virus production. Error bars represent observed range, n = 2.

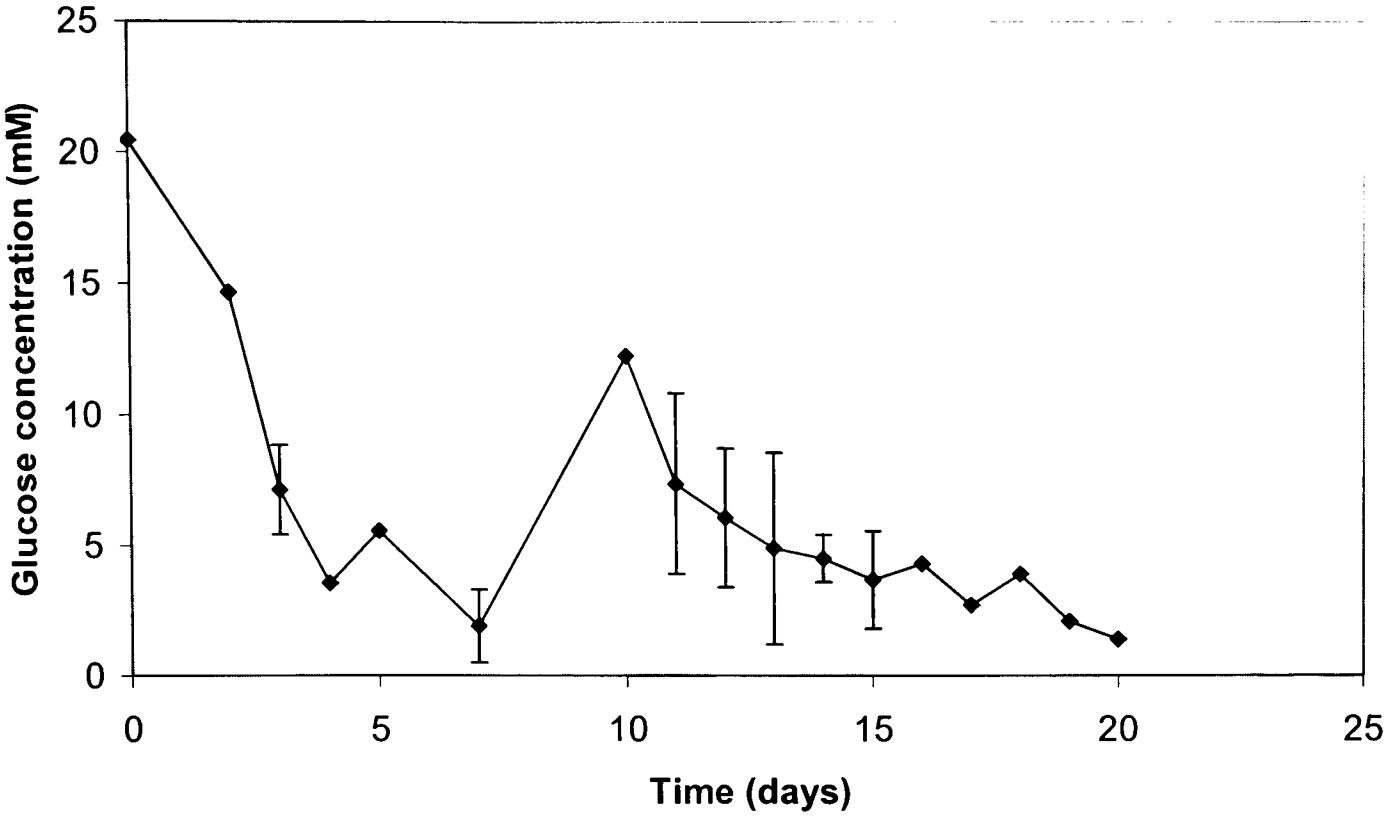


Figure 4.3.2.6

The residual glucose concentration for TEFLYED/83 cells grown in a packed bed bioreactor with internal aeration. The total working volume was 200ml. Medium was changed after 4 days, 6 days and daily after 9 days. Error bars represent observed range, n = 2.

In addition to the packed bed systems, cells were cultivated on Immobasil FS microcarriers in 100ml spinner flasks. Immobasil has previously been shown to be a suitable substrate for human packaging cell lines producing retrovirus particles (McTaggart and Al Rubeai 2000). The ability of stirred cultures to efficiently produce virus particles is doubtful (Gerin *et al.* 1999). In this study, the cell number showed a dramatic increase during the initial 24 hours, prior to the onset of agitation. The cell number fell to ~50% of the inoculum within 24 hours of the commencement of stirring but recovered during the following 6 days to reach a maximum number of 7.1 x 10^7 cells. This is equal to a cell density of 7.1 x 10^6 cells ml^{-1} carrier. During the production phase the cell number gradually decreased, with a final cell number of 4.14 x 10^7 cells (see Figure 4.3.2.7). This suggests that stirred cultures are unable to support long-term cultivation of TEFLYRD/83 cells at 32°C.

Cell attachment and growth on microcarriers is dependent on several parameters including agitation speed, culture pH, inoculum ratio, bead concentration and serum concentration (Forestell *et al.* 1992; Hu *et al.* 1999; Mendonça *et al.* 1999; Ng *et al.* 1996; Shigami *et al.* 1997). The inoculum ratio and bead concentration used were those recommended by the manufacturer. The culture medium was supplemented with 10% serum. This did not have any adverse affect on cell attachment kinetics and 89% of cells had disappeared from suspension after 1 hour.

The initial 24 hours of static conditions allowed cells to grow and expand into the matrix. The majority of the cells remained on the outer surface of the carriers. Hence, when agitation was started these cells were subjected to mechanical stress, which eventually led to cell death. The remaining cells were able to colonise the internal surface of the particles and reach a maximum cell density of 7.1 x 10^6 cells ml^{-1} carrier. However, this was considerably lower than densities reported for the cultivation of other cell lines and reiterated the limited potential of stirred cultures for

the cultivation of this cell line. Cell growth was not limited by glucose utilisation, as shown in Figure 4.3.2.8.

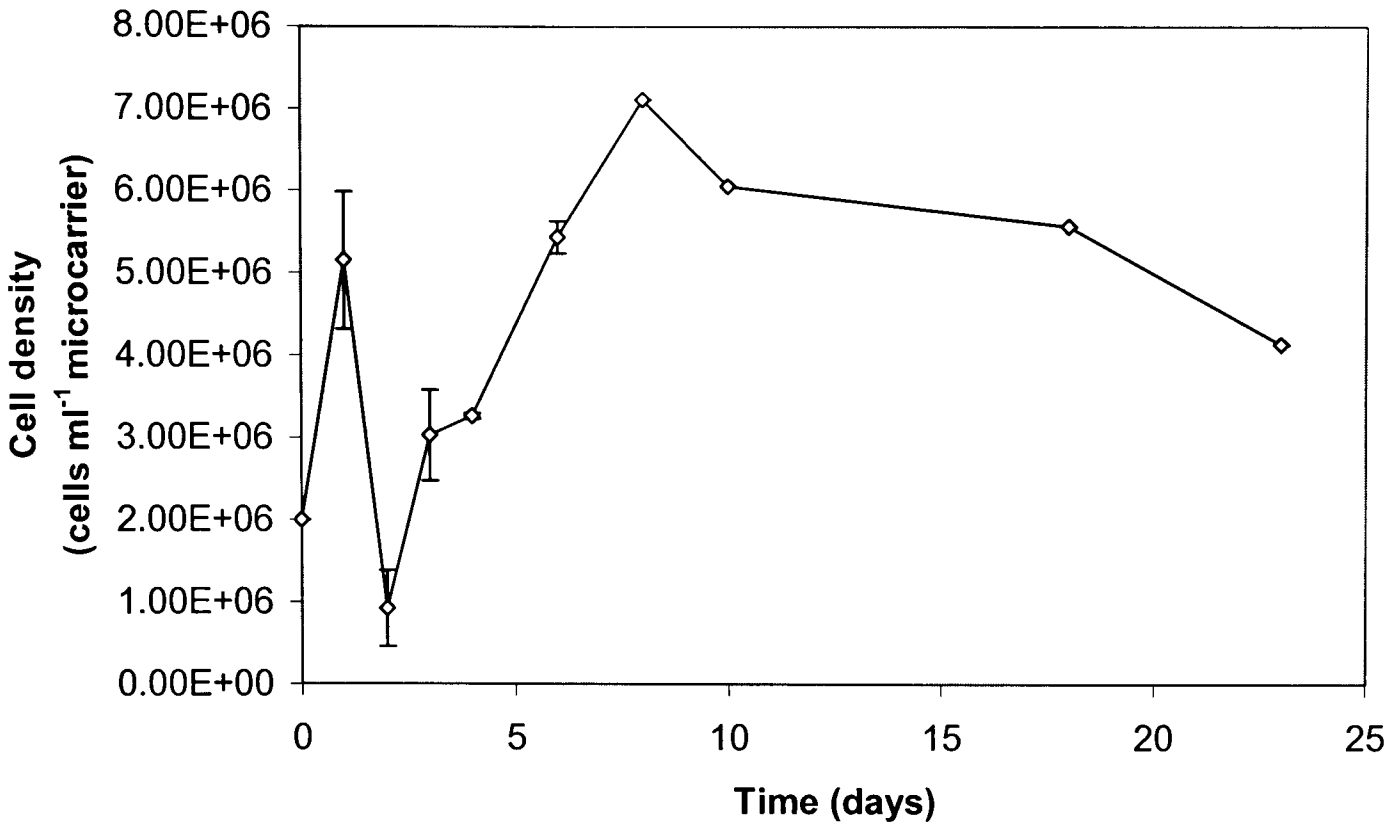


Figure 4.3.2.7

Mean cell density for TEFLYRD/83 cells in 100ml spinner flask culture on Immobasil FS beads. Agitation was initiated after 24 hours. The culture temperature was reduced from 37°C to 32°C after 10 days. Error bars represent observed range, n = 2.

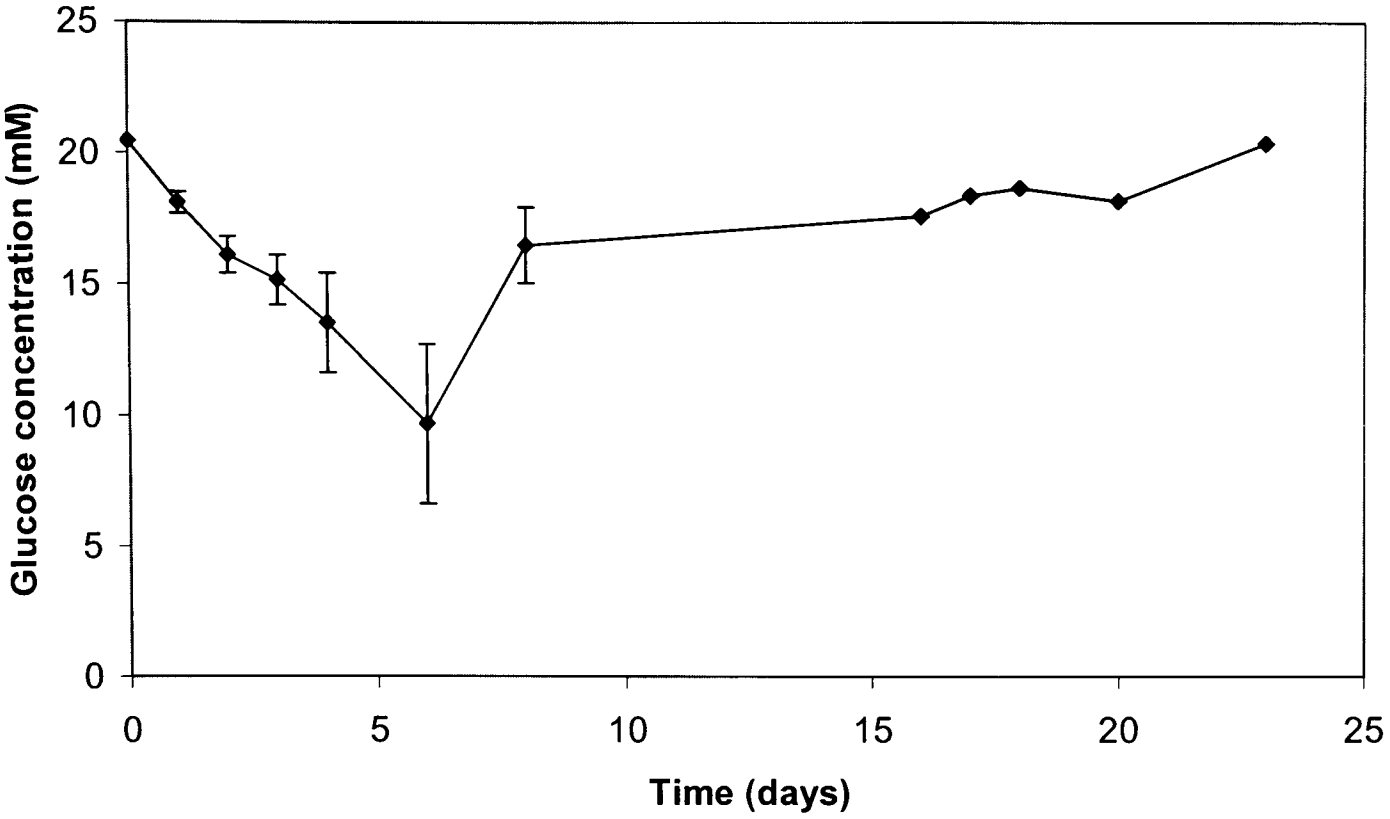


Figure 4.3.2.8

The residual glucose concentration for TEFLYRD/83 cells in 100ml spinner flask culture. Medium was changed after 6 days, 8 days, and then daily after 10days. Error bars represent observed range, n = 2.

4.3.3 Retrovirus production

The retrovirus titre from duplicate experiments was measured on day 1, day 5 and day 10 of the production phase (after cells had been acclimatised to 32°C) using RT-PCR. The average daily retrovirus production in roller bottles was calculated to be 1.48 x 10^7 LacZ Transforming Units (LTU) ml^{-1} day^{-1}. The maximum virus titre was 1.95 x 10^7 LTU ml^{-1} and the total production during the course of the culture was estimated to be 7.4 x 10^9 LTU. The cell specific production rate was calculated as 4.09 LTU $cell^{-1}$ day^{-1}. Hence, roller bottles provide a favourable environment for high titre virus production.

The most promising alternative to roller bottle culture was the PBR. The average daily vector production was 9.24 x 10^6 LTU ml^{-1} day^{-1}, and was significantly higher than production from the spinner basket or spinner flask cultures. However, productivity in the roller bottle culture was 60% higher than in the PBR. The advantage of the PBR is that it can produce a large amount of virus supernatant in a relatively small space. During the course of the culture the PBR produced a total of 1.8 x1010 LTU. This is equivalent to 2.4 roller bottles. Another promising feature of this system is the specific production rate was 3.23 LTU $cell^{-1}$ day^{-1}. While this is still lower than the specific production rate in roller bottles, it is higher than the other production systems evaluated in this study. It is believed that optimising the culture conditions as described in the above section can further increase this production rate.

The level of productivity in the spinner basket was not satisfactory, when compared to roller bottle culture or the PBR. The average productivity rate was calculated as 3.44 x 10^6 LTU ml^{-1} day^{-1}, which produced a cumulative virus titre of 1.38 x 10^{10} LTU. From the three different methods that were evaluated and compared to roller bottle culture, it was anticipated that the spinner basket would produce the highest amount

of total virus due to the fact that it had the largest culture volume and the potential to support the highest number of cells. Unfortunately, this was not the case as the specific production rate of the cells was 0.778 LTU $cell^{-1}$ day^{-1}. Despite having the largest total cell number at the end of the culture, the cells were unable to produce high titre virus supernatant.

The daily virus titres measured from the spinner flask culture showed a decrease during the course of the production phase.

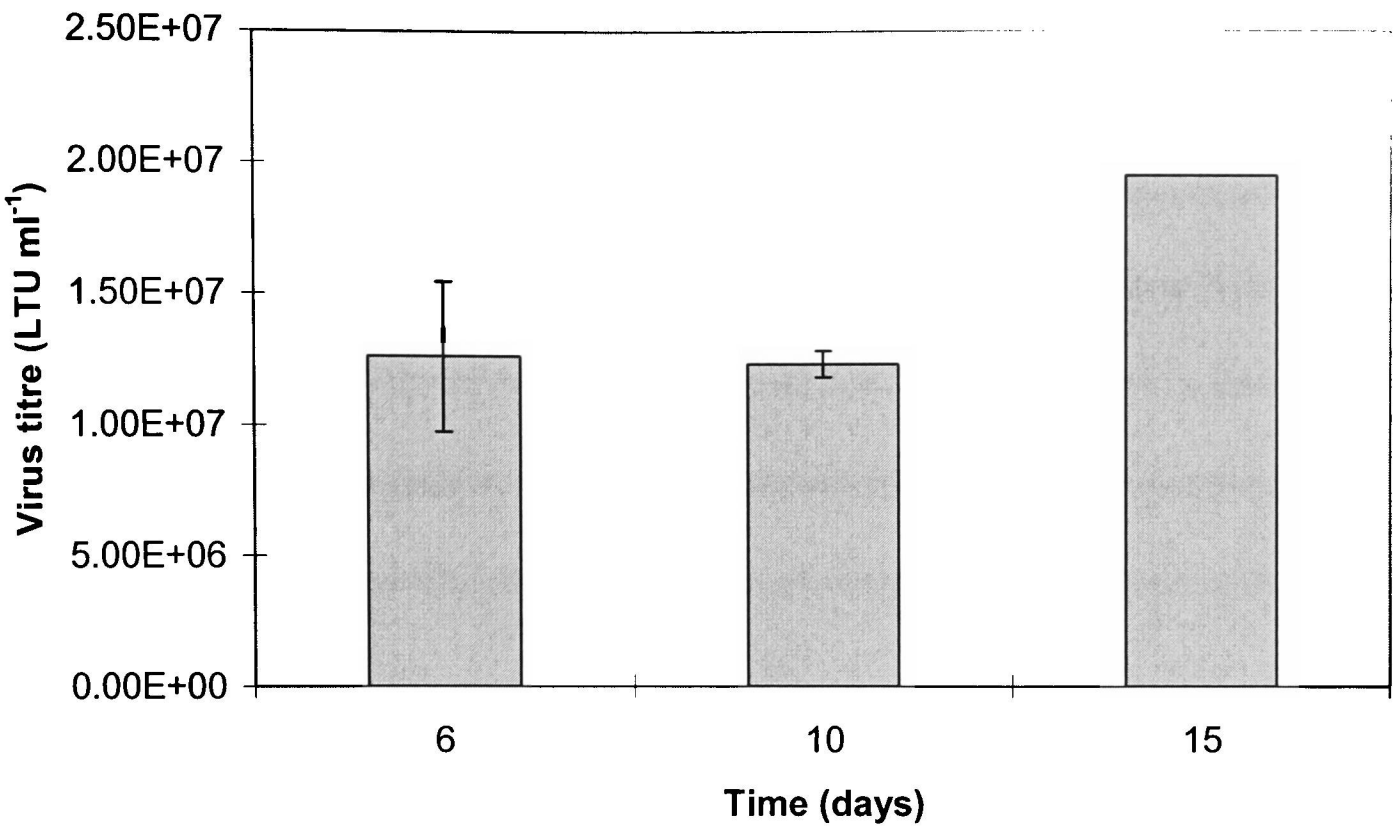


Figure 4.3.3.1

Virus titre at 32°C for TEFLYRD/83 cells cultured in roller bottles. The virus titre was measured from harvested supernatant from the respective days using RT-PCR. The samples were stored in liquid nitrogen prior to analysis. Error bars represent observed range, n = 2.

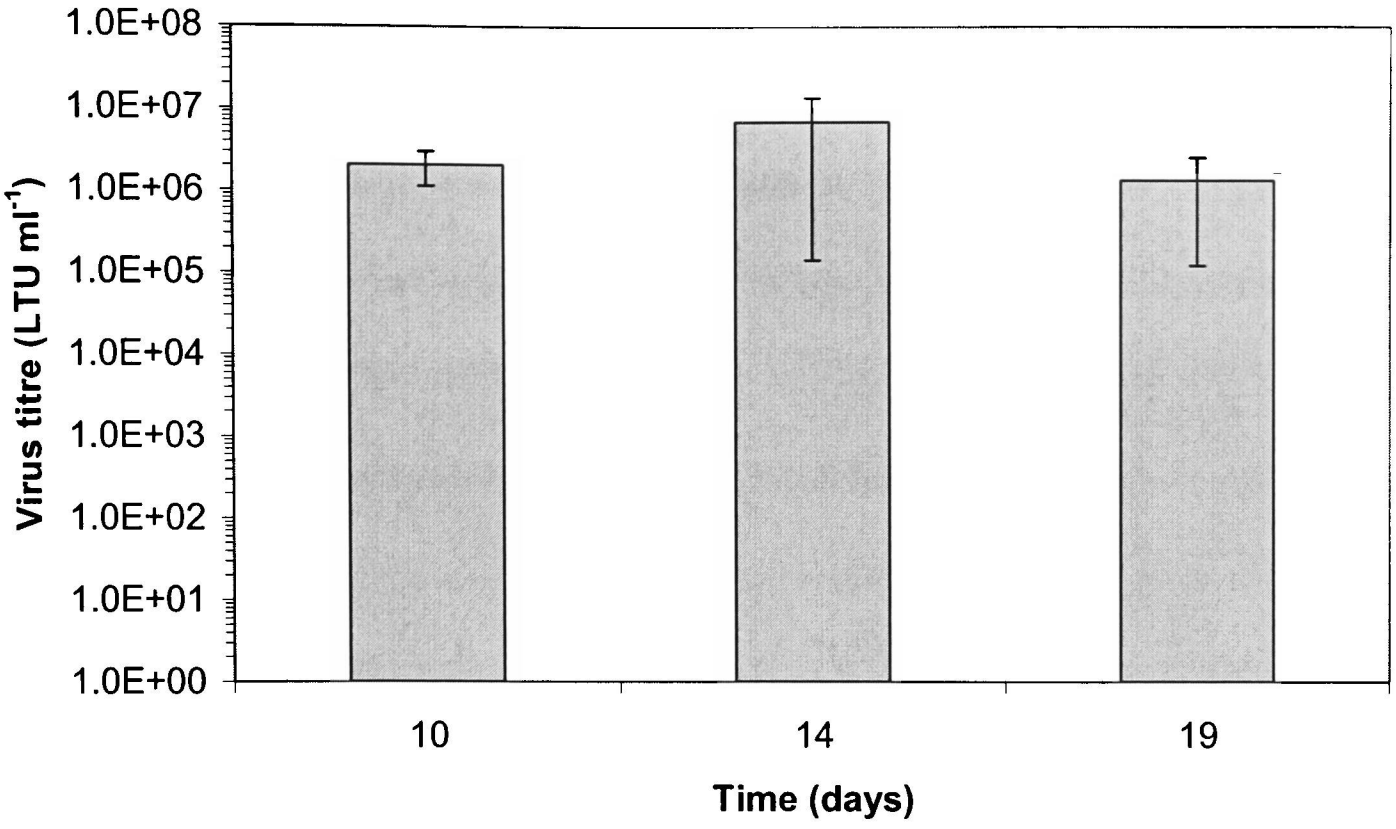


Figure 4.3.3.2

Virus titre at 32°C for TEFLYRD/83 cells in a spinner basket packed bed bioreactor. Following the reduction of the temperature from 37°C to 32°C, a complete medium change was performed daily and 1ml of virus containing supernatant was stored in liquid nitrogen for later analysis. Virus titre was determined by RT-PCR from duplicate samples. Error bars represent observed range, n = 2.

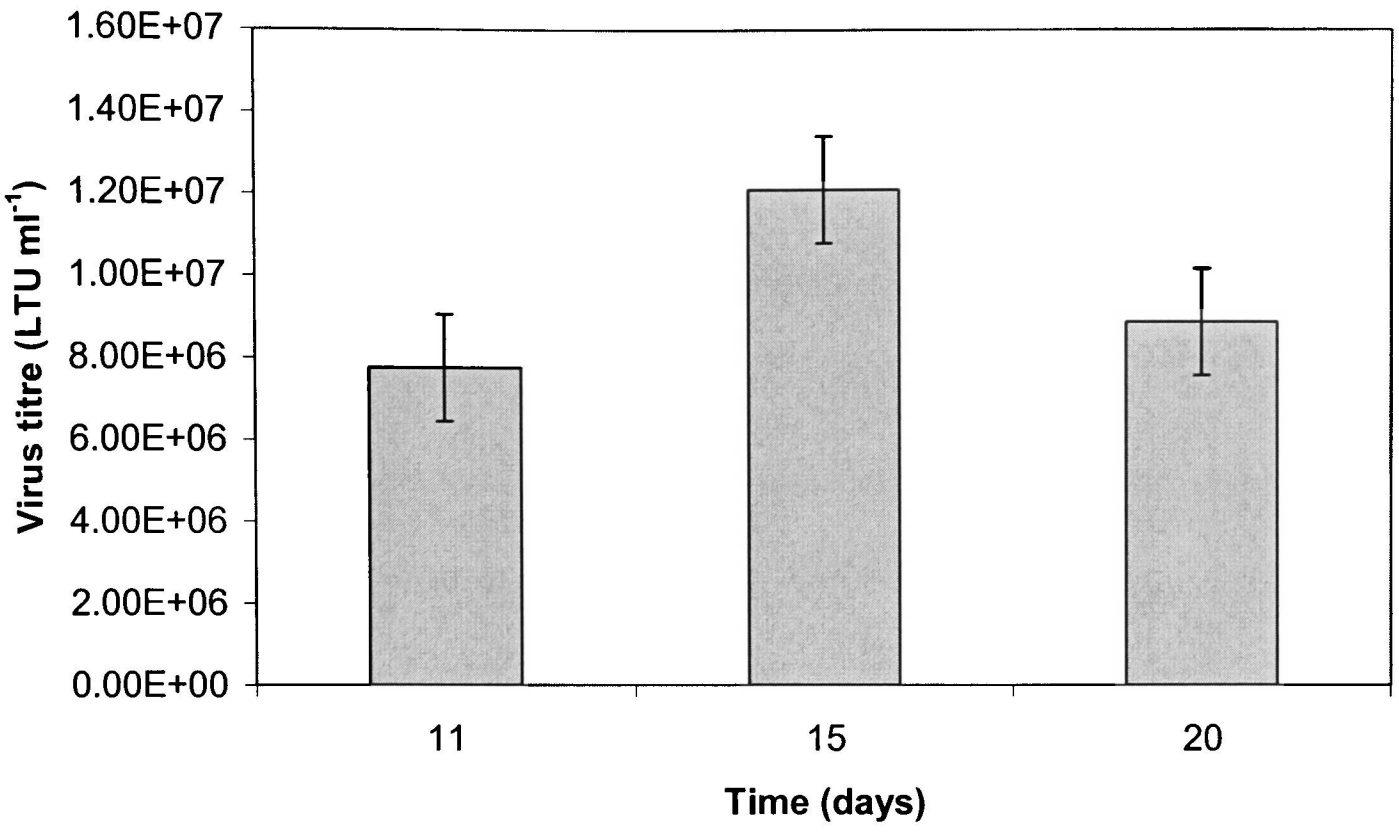


Figure 4.3.3.3

Virus titre for TEFLYRD/83 cells in a packed bed bioreactor with internal aeration at 32°C. A daily medium change was performed from day 10 onwards, after the culture temperature had been reduced from 37°C to 32°C. 1ml of the harvested medium was stored in liquid nitrogen and the virus titre was measured upon completion of the experiment using RT-PCR. Error bars represent standard error.

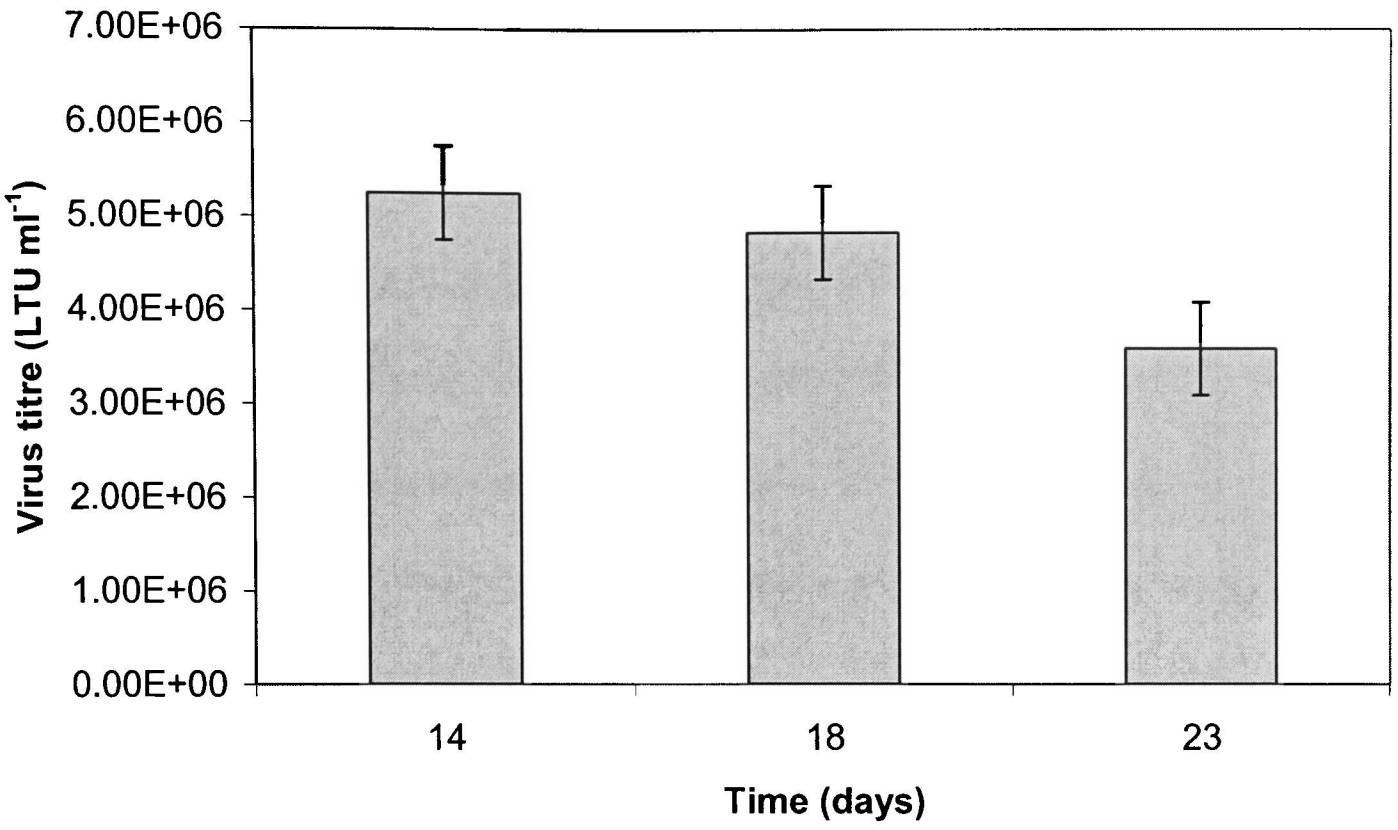


Figure 4.3.3.4

Virus titre for TEFLYRD/83 cells in 100 ml spinner flask culture with Immobasil FS microcarriers at 32°C. 10ml of carriers were cultured in 90ml of culture medium. Once cells had reached the stationary phase the culture temperature was reduced from 37°C to 32°C. The medium was changed at regular intervals to prevent glucose limitation and 1ml of virus containing supernatant was periodically collected and stored in liquid nitrogen. The virus titre was measured using RT-PCR. Error bars represent standard error.

It is thought that there are multiple factors that account the differences in retroviral titres and the specific productivity. A common feature of roller bottles and PBR is that over the 24 hour period between medium changes, the residual glucose fell below 5.5mM. Nutrient deprivation or metabolite accumulation does not have a direct limitation on virus production (Gerin *et al.* 1999a). It will, however, have an indirect affect on virus production, as cell propagation and nutritional state will be effected.

It is well reported that virus production is dependent on cell proliferation. Although virus assembly is not predominant in any particular phase of the cell cycle it is essential that cells progress through the cycle (McTaggart and Al Rubeai 2001). Viral replication requires the 5′ and 3′ long terminal repeats (LTRs) as these contain promoter and enhancer sequences (Kim *et al.* 2000; Smith 1995). LTRs are most active in cycling cells (Merten *et al.* 2001), therefore, virus productivity will be enhanced in cultures where cells are continuously dividing. It was observed that the supernatant from roller bottle cultures contained noticeable amounts of cell debris. This demonstrates that although the viable cell number remained constant throughout the production phase, cell death was prevalent with dividing cells replacing those that were lost. This active cell division is one factor that accounts for the increased specific production rate. Similarly, in the PBR the GUR increased throughout the course of the production phase, which may be explained by an increasing cell number. Hence, cells were actively dividing in the PBR during production. The lower specific production rates observed in the spinner basket may have been the result of a reduced cell proliferation rate. We have deduced that cells were in sub-optimal conditions by their enhanced requirement for glucose and that this was primarily used for maintenance energy, as oppose to growth energy. This would result in a reduction in the expression of LTRs, which leads to lower specific virus production. In the spinner flask, the cell number gradually decreased over the course of the production phase,

showing that cell death was the dominant feature in these cultures, and the death rate was greater than the growth rate.

Another factor that may affect virus production is the type of microcarrier used. Fibra-Cel™ has proved to be a more favourable substrate than Immobasil for the production of retrovirus particles (see chapter 3). This is due to the microenvironment surrounding cells immobilised within the particles. The carriers are constructed of different materials which will determine cell spreading/aggregation behaviour and the geometric arrangements in the matrix. This will influence the transport of nutrients to the cells and the release of virus particles into the culture supernatant. The restriction of nutrients to cells in the centre of the carriers will result in a poor nutritional state, where cells are unable to produce virus, and may lead to a necrotic core in the beads. Necrotic cells release proteases, DNA and other cellular components (Fassnacht *et al.* 1999) which will affect the surrounding cell population and may have a negative effect on the stability of virus particles. Immobasil FS carriers have a spherical geometry, which means that any cells that spread into the internal structure will be susceptible to nutritional gradients. As mentioned earlier, cells are unable to occupy the external surface of the carrier due to hydrodynamic forces. Therefore, it is hypothesised that cells only remain viable in a thin shell close to the surface of the carrier, thus only low cell densities are attained and productivity is relatively low. In contrast, the disc shape of Fibra-Cel™ allows cells to propagate the entire structure as the distance for nutrient diffusion is relatively small through the cross-sectional plane. In addition to the favourable geometry of disc microcarriers, diffusion of nutrients through packed bed bioreactors is enhanced by intraparticle convective flow. In monolayer culture the virus particles are assembled and released into the supernatant without any resistance. In microcarrier culture the particles have to diffuse to the exterior. The increased residence time within the matrix will have a negative effect on

virus harvest due to the short half-life of the retrovirus. The intra-particular convective flow through a packed bed not only allows greater nutrient transport but also for vector release. The inefficient fluid flow through the bed of the spinner basket may be insufficient to effectively transport virus particles from the basket into free supernatant. However, the fluid flow through the PBR was capable of not only providing adequate amounts of nutrients to cells but also of carrying virus particles to the medium reservoir.

In the spinner flask and spinner basket hydrodynamic forces were present due to the agitation within the culture vessel. The shear stress generated from the impellers was not deemed to be a cause of virus damage. Retrovirus particles have on average a diameter of 100nm (Andreadis *et al.* 1999), whereas the smallest eddies present in a spinner flask culture are in the range of 170-200μm. Therefore, virus particles would simply be entrained by eddies and would not suffer any adverse effects from particle-eddy interactions.

Once virus particles have been released into the culture medium they are subject to several environmental factors that may have an effect on their stability. Viral vector stability can be influenced by factors such as temperature, pH and incubation conditions such as growth medium and serum components (Higashikawa and Chang 2001). In order to allow a pragmatic comparison between culture systems, conditions were kept constant i.e. the temperature was maintained at 32°C and identical culture medium and serum was used. However, it was not possible to regulate the pH or dissolved oxygen in these systems. CO_2 is a product of respiration and glucose metabolism which affects the pH of the culture medium causing it to become more acidic. The medium did contain sodium bicarbonate but this was not considered an effective buffer. Therefore, differences in pH between systems may have affected the amount of virus harvested as a dramatic decrease in vector half-life is observed at pH

levels below 7.0. Although pH was not measured in this study, it has been reported that it can drop to 6.5 over a 24 hour period in high density cultures (Higashikawa and Chang 2001; McTaggart and Al Rubeai 2000).

4.3.4 Comparison of perfusion culture systems

Continuous perfusion culture systems offer many advantages for the long-term production of virus particles when compared to semi-continuous cultures. The continually feeding of medium to the culture vessel will eradicate the problem of nutrient limitation whilst continual removal of waste products prevents the accumulation of toxic metabolites. Virus particles can be efficiently removed from the culture vessel to a harvest vessel at lower temperature to increase the stability of particles. In the systems tested the bioreactor conditions could be controlled, ensuring that pH and dissolved oxygen levels were maintained at acceptable levels. The four systems tested were the stirred tank bioreactor, the packed bed bioreactor with internal aeration, a fixed bed bioreactor and a fluidised bed bioreactor. Due to the different method used to determine the virus titre it was not possible to directly compare these results with those obtained for semi-continuous cultures at 32°C.

4.3.5 Cell growth and metabolic trends

The standard stirred tank bioreactor is the technology of choice for the industrial production of the majority of biopharmaceutical products (Chu and Robinson 2001). This makes it an attractive option in process development as many issues pertaining to regulation and licensing are more easily overcome than with other methods. In this study, Cytoline 1 microcarriers were used in the stirred tank bioreactor partly because of the unsatisfactory results obtained with Immobasil FS microcarriers in spinner flask culture and also to allow a better comparison with the fluidised bed bioreactor, which used Cytoline 1 carriers.

The cell density in stirred tank bioreactors showed no definite increase during the course of the experiments as depicted by the GUR in Figure 4.3.5.1. In the first run no viable cells were detected after 5 days and a constant glucose concentration indicated that there was no evidence of cell growth. During the second run cells were maintained for 12 days. After this time the increase in glucose concentration indicated a complete loss in cell viability. In both cases the residual glucose concentration never fell below 10mM and therefore perfusion was not initiated (see Figure 4.3.5.2).

The loss of cell viability was attributed to the stirring regime employed in the bioreactor and the type of microcarrier used. In previous experiments Cytoline 1 carriers have proved very effective for the growth of TEFLYRD/83 cells. However, as these carriers were originally designed for use in fluidised bed reactors their density is greater than other macroporous carriers designed for use in stirred tank reactors. This higher density requires greater agitation to fully suspend the beads and obtain a homogenous environment. As stated by the Kolmogorov theory, the microscale of turbulence is a function of energy dissipation and kinematic viscosity. Amongst the parameters used to calculate the energy dissipation are the agitation speed and the power number of the impeller. The STR was fitted with a 6-bladed rushton turbine with a power number of 5 (*c.f.* power number of the spinner flask was 0.5). These two factors are thought to account for the difference in performance observed between spinner flask culture and the stirred tank bioreactor. Another consideration are the relatively large pores of Cytoline 1 microcarriers (10-400 μm), which may not offer adequate protection of cells from these mechanical forces, especially as the eddy sizes would be less than some of the pores.

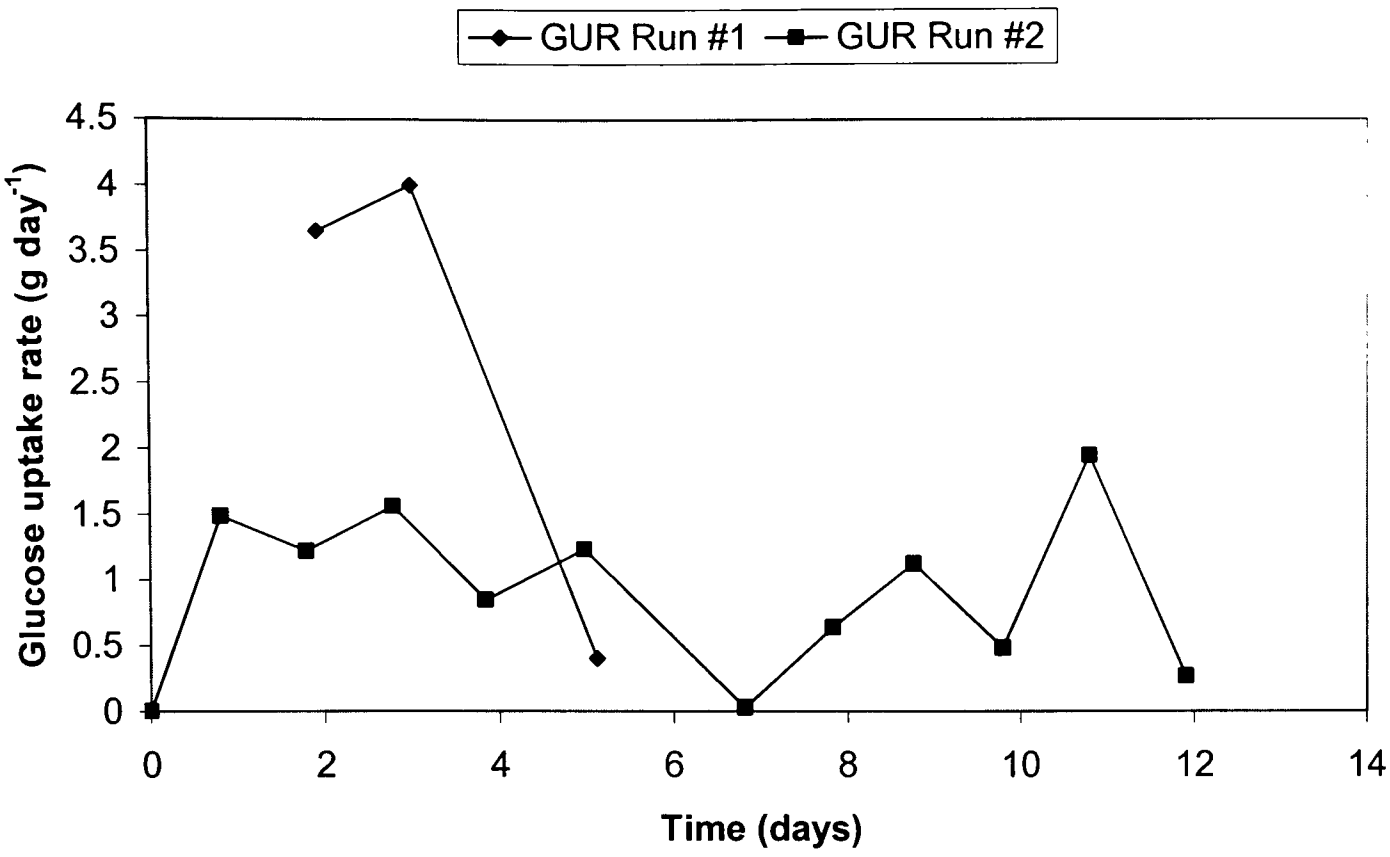


Figure 4.3.5.1

The glucose uptake rate in a perfusion culture of TEFLYRD/83 cells, cultured in a stirred tank bioreactor. Cells were attached to Cytoline 1 microcarriers (100ml) in 900ml of culture medium. The bioreactor set points were: temperature 37°C; DO_2 50%; pH 7.0; agitation speed 100 rpm. Medium samples were collected at regular intervals and the glucose concentration was measured off line using a GLUCOTREND® glucose test kit. The GUR was calculated from the change in residual glucose concentration.

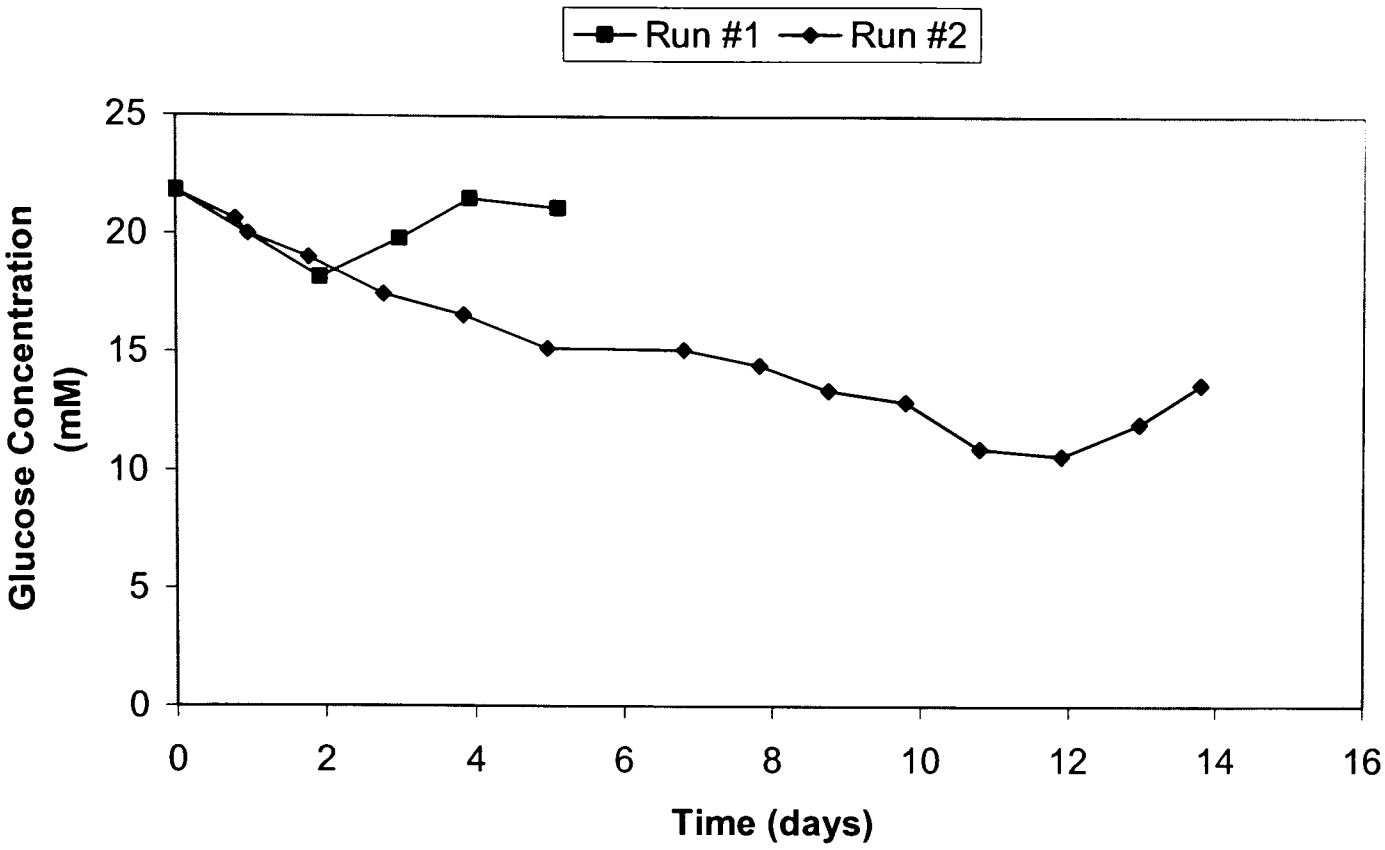


Figure 4.3.5.2

Residual glucose concentration for a perfusion culture of TEFLYRD/83 cells cultured in a stirred tank bioreactor. Medium samples were collected at regular intervals and the glucose concentration was measured off line using a GLUCOTREND® glucose test kit. Perfusion of culture medium was not started as the residual level didn't fall below 10mM.

The packed bed bioreactor (PBR) has shown a great deal of promise in semi-continuous cultures. In order to improve performance the feed medium was conditioned in a stirred tank bioreactor and the virus containing supernatant was harvested in a separate vessel at a lower temperature. The difference in cell numbers obtained between duplicate experiments highlights the difficulties in obtaining reproducible results with this culture system. In the initial run cells were able to grow steadily over the course of 16 days. The maximum cell number achieved was ~1 $x10^8$ cells which equates to 2 x 10^6 cells ml^{-1} carrier. In the second run cell growth was extremely rapid during the initial stages and a cell number of >4 x 10^8 cells was reached within 4 days (see Figure 4.3.5.3). However, this cell number was not maintained as the perfusion rate was increased and after 21 days there was no evidence of cell viability.

In both experiments the perfusion rate was determined by the concentration of residual glucose. Where the concentration of glucose increases the perfusion rate is higher than the nutritional demand of the cells. This is expected immediately after an increase in perfusion rate but it was anticipated that the concentration would gradually decrease as cell numbers increased. As cells did not continue to multiply the glucose concentration remained unchanged as illustrated in Figure 4.3.5.4.

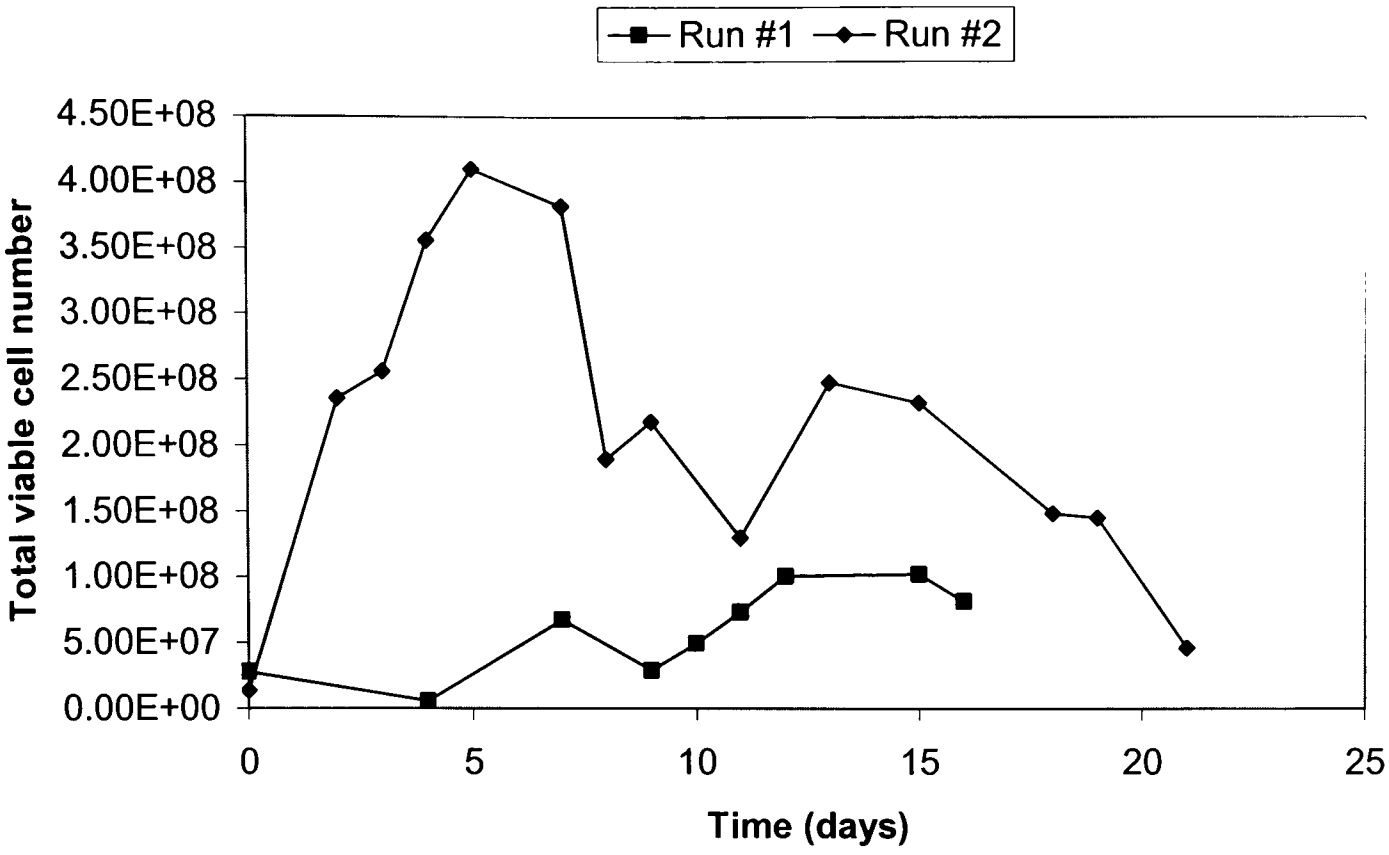
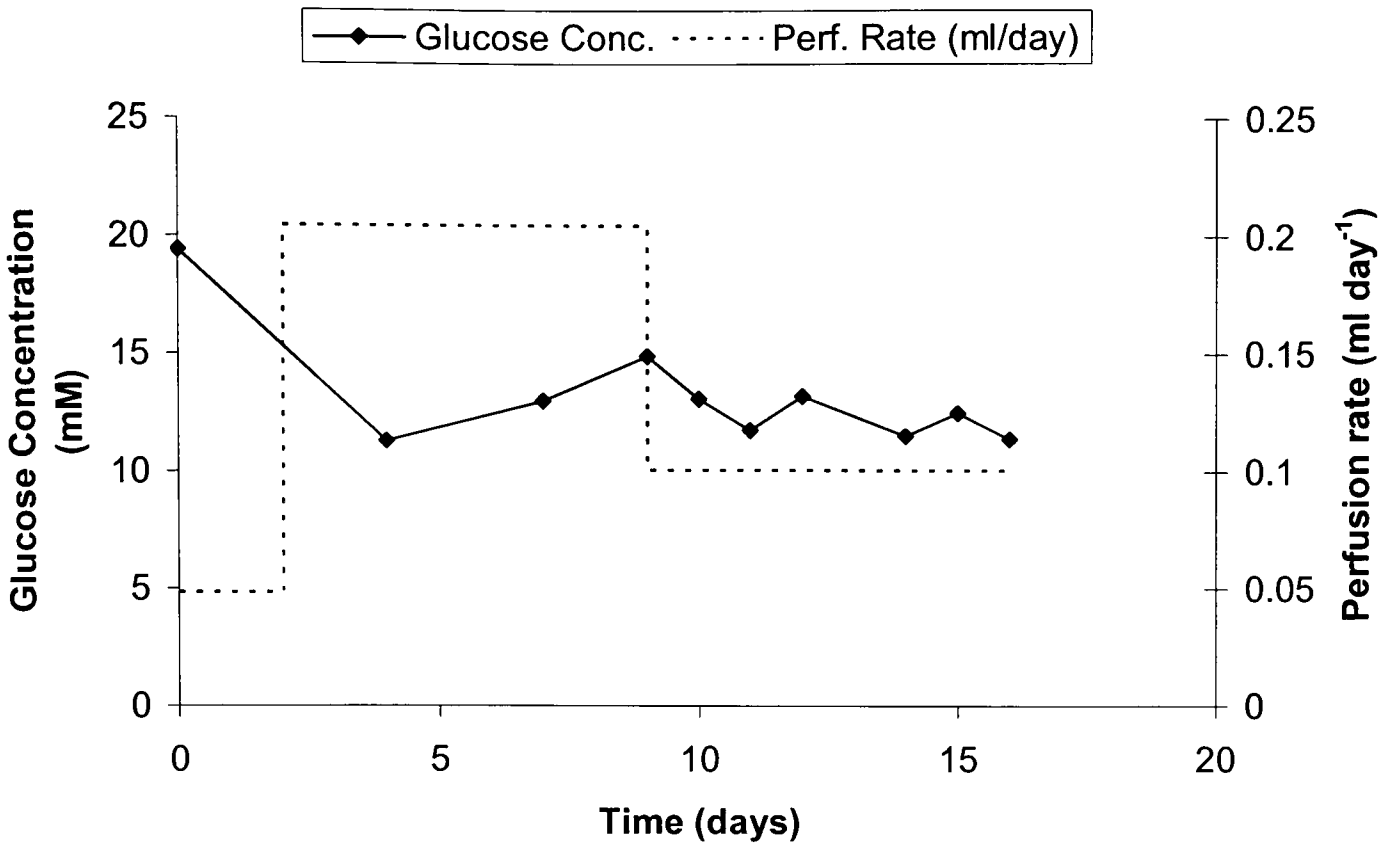


Figure 4.3.5.3

The viable cell number of an open perfusion culture of TEFLYRD/83 cells in a packed bed bioreactor with internal aeration. Cell numbers were calculated at the end of the experiment using the MTT assay. Intermediate cell numbers were estimated from the GUR due to the inaccessibility of cells immobilised within the vessel. Medium samples were collected at regular intervals and the glucose concentration was measured off line using a GLUCOTREND® glucose test kit. The GUR was calculated from the change in residual glucose concentration.

Medium was circulated through the packed bed using a peristaltic pump. The culture set points were temperature 37°C; DO_2 75%; pH 7.0.

A



B

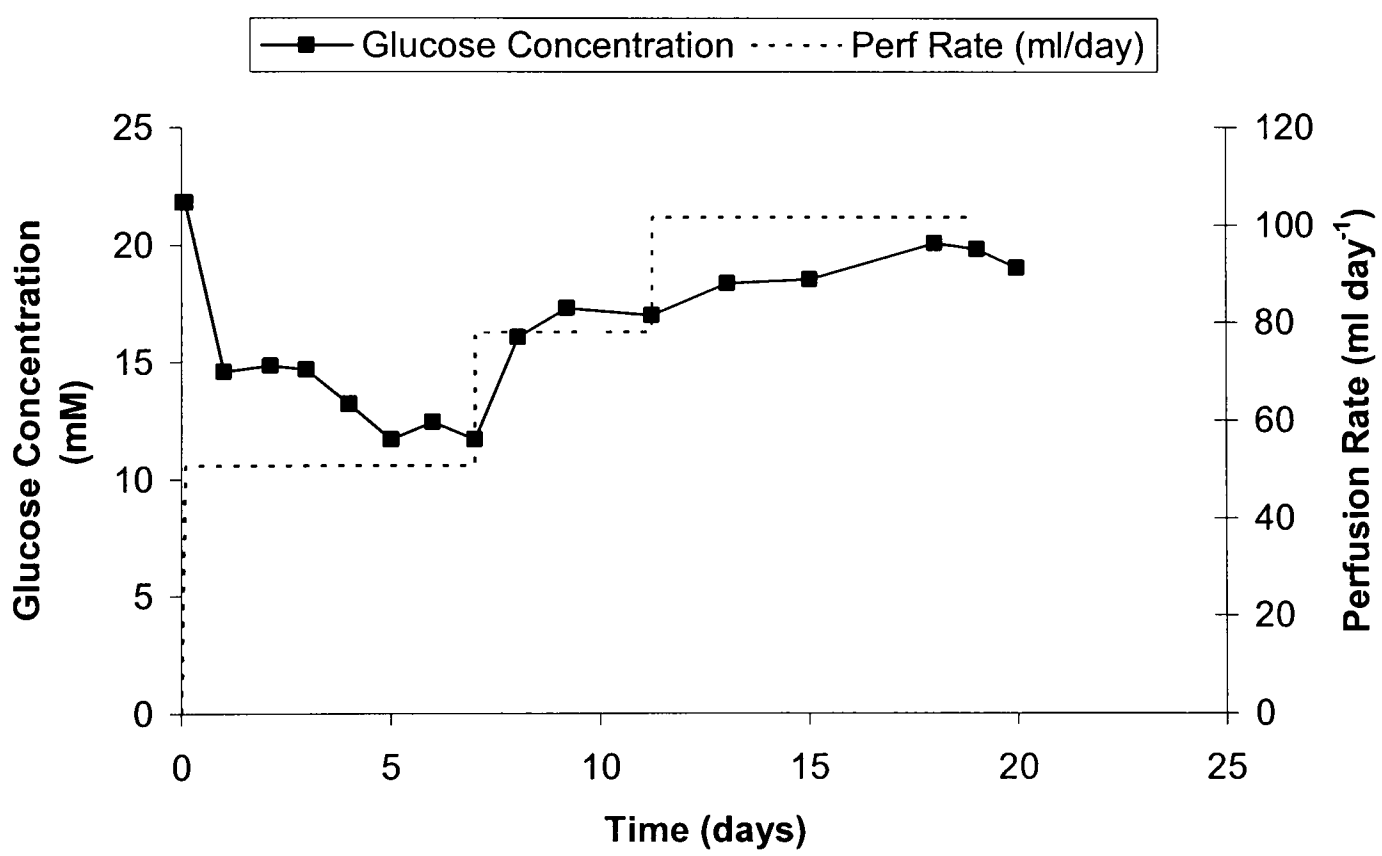


Figure 4.3.5.4

Residual glucose concentration and perfusion rate in an open perfusion culture of TEFLYRD/83 cells cultured in the packed bed bioreactor. A = run #1, B = run #2. The perfusion rate was adjusted according to the residual glucose concentration.

The fixed bed bioreactor proved an effective method for the long term cultivation of TEFLTRD/83 cells. The first run was ended after 16 days due to complete consumption of the feed medium. During the second run it was possible to maintain the culture for 28 days as can be seen from Figure 4.3.5.5. Although long-term cultivation was possible, the cell density did not increase to anticipated levels. For the majority of the experiment the cell density was ~3.3 x 10^6 cells ml^{-1} microcarrier and the maximum density reached during run #2 was 4.8 x 10^6 cells ml^{-1} microcarrier.

As with previous perfusion cultures, the glucose concentration was used as a parameter for determining the perfusion rate. The residual glucose concentrations and perfusion rates for each run are shown in Figure 4.3.5.6. The constant medium recirculation between the fixed bed compartment and the conditioning vessel allowed effective uptake of glucose and oxygen, as demonstrated from the changes in residual glucose concentration and the requirement for constant sparging. As the glucose was depleted in the conditioning vessel, so the perfusion rate was increased. It was only towards the end of the second run that glucose concentration began to increase within the vessel, and this was attributed to a gradual decline in cell number, caused by the longevity of the culture.

Despite the disappointing cell densities the fixed bed bioreactor has shown good potential for the cultivation of TEFLYRD/83 cells and warrants further investigation and optimisation to enhance cell growth.

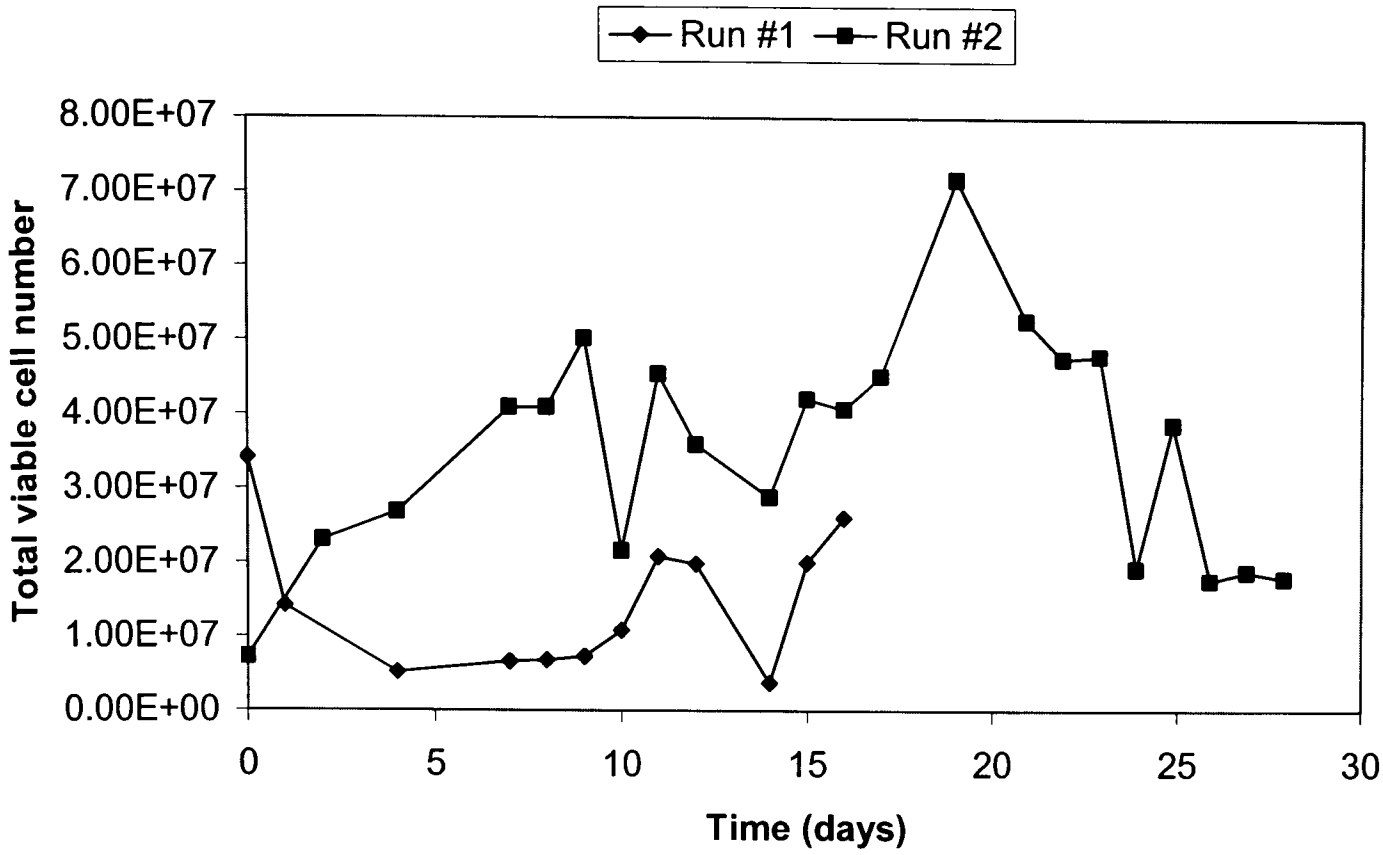
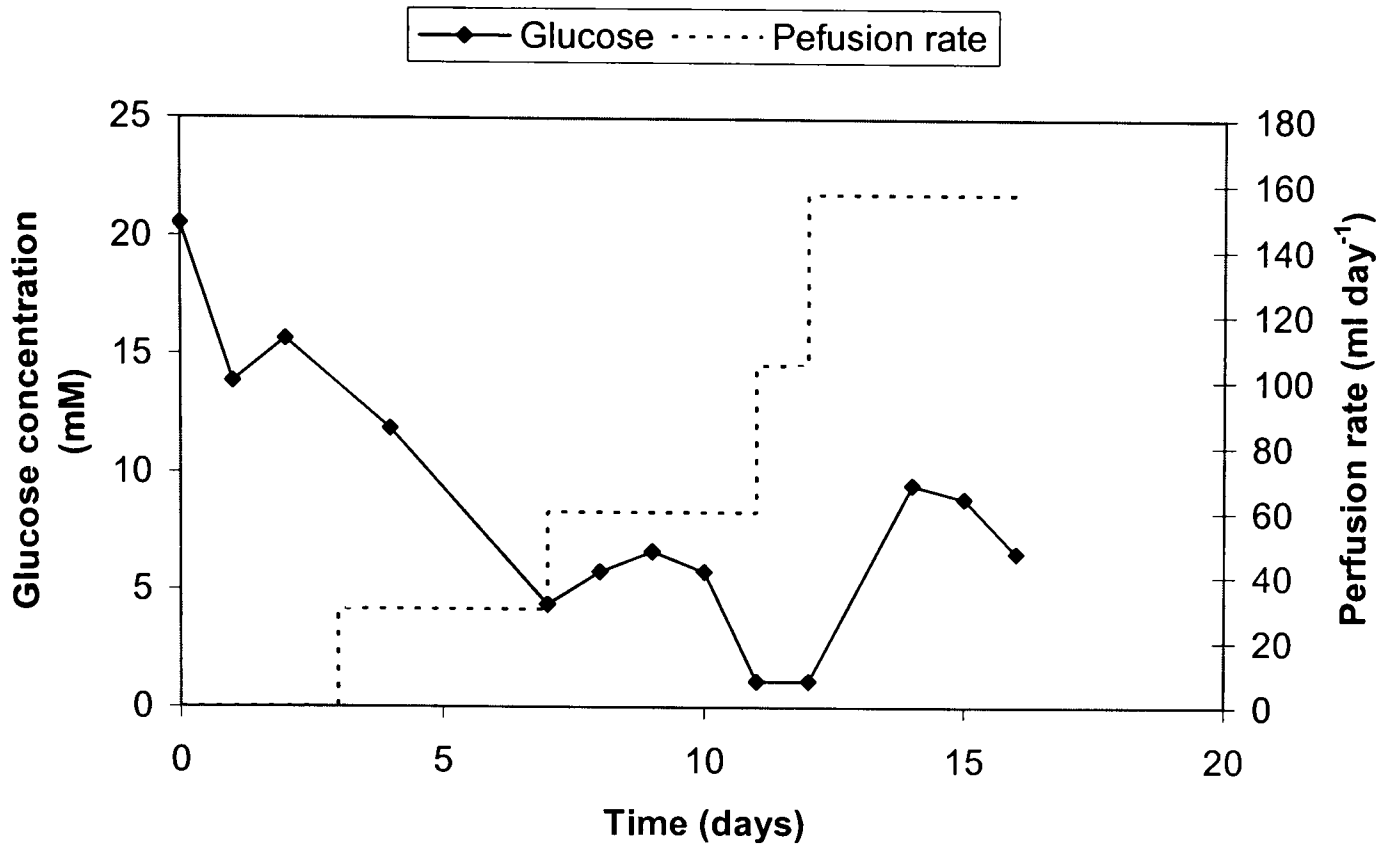


Figure 4.3.5.5

The viable cell number of an open perfusion culture of TEFLYRD/83 cells in a fixed bed bioreactor. Medium was circulated between the conditioning vessel and the fixed bed. Cell numbers were measured at the end of the experiment using the MTT assay. Intermediate cell numbers were estimated from the GUR due to the inaccessibility of cells immobilised within the vessel. The culture set points were temperature 37°C; DO_2 50%; pH 7.0. Medium circulation between the fixed bed and the conditioning vessel was started 3 hours after inoculation at a rate of 1ml min^{-1}. This was increased to 5ml min^{-1} during the following 5 days.

A



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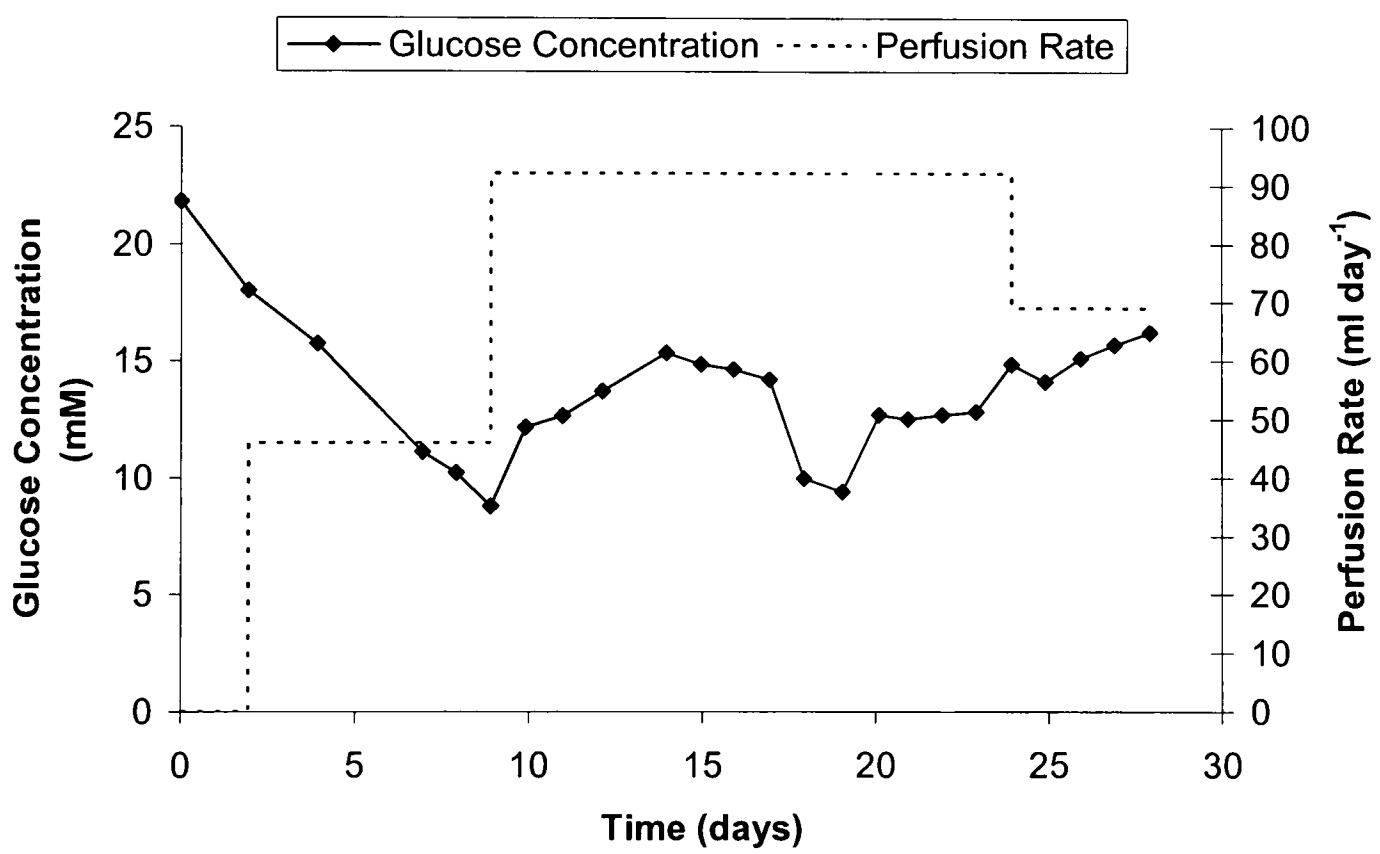


Figure 4.3.5.6

Residual glucose concentration and perfusion rate in a perfusion culture of TEFLYRD/83 cells cultured in a fixed bed bioreactor. A = run #1, B = run #2. The perfusion rate was adjusted according to the residual glucose concentration.

Due to the potential problems of scalability with packed bed reactors, the feasibility of cell growth and retrovirus production was examined in a fluidised bed bioreactor. This system is manufactured up to a scale of 400L by Vogelbusch (Germany).

The culture was run over a period of 19 days. During this time a total cell number was reached of $1.58 \pm 0.25 \times 10^9$ cells (see Figure 4.3.5.7). This shows an overall increase of 3-4 fold. Interestingly, the initial cell number used in run #2 was considerably lower than that used in run #1, yet the final cell numbers were similar. This suggests that cell division will be more rapid at lower densities and that the total cell number is controlled by the residual glucose concentration.

During the course of the experimental runs the glucose concentration was maintained above 8.5mM by adjusting the perfusion rate according to the residual concentration as shown in Figure 4.3.5.8. This ensured that cell growth was not limited at any point during the culture.

The final cell numbers were disappointing, though. The cell density was 5.27×10^6 cells ml^{-1} microcarrier. In the manufacturer's literature it is stated that cell densities of 2×10^8 cells ml^{-1} microcarrier are achievable, yet this was not found to be the case with TEFLYRD/83 cells. This problem is believed to be the result of poor oxygen transfer to cells entrapped within microcarriers. The GUR and the glucose concentration within the vessel do not suggest that any nutritional limitations were present. Alternatively, poor cell density may be because the cell line does not like to maintain cell division on such material. However, a recent study on the poor performance and productivity of a fluidised bed bioreactor under similar conditions, were attributed to oxygen limitations (Preissmann *et al.* 1997). They reported that the maximum penetration depth of oxygen was 300μm and that 40% air saturation could not provide efficient oxygenation of the surface biofilm during the exponential phase. Whilst oxygen uptake rate or oxygen transfers were not examined in our study the

results appear to conform to the findings of Preissmann *et al.* (1997). Therefore, oxygen supply is believed to be the limiting factor for cell growth and explains the poor performance.

Another draw back of this system, and the reason why the culture was halted after 19 days, was that microcarriers began clumping. This had a detrimental effect on the level of fluidisation and agitation speed had to be increased to maintain 100% fluidisation of the bed. There are two possible reasons why clumping occurred. The first is that the serum in the medium bound to the surface of the carriers and caused them to stick together. The problem could be overcome by gradually reducing the serum concentration in the culture during the course of the experiment. Serum is required by the cells for growth and attachment and so a reduction in the initial serum concentration may not be practicable, nor would a complete removal of serum towards the latter stages of the culture, although these need further investigation. The second explanation for microcarrier clumping is the bridging of cells. This phenomenon occurs when cells attach to more than one microcarrier, forming an aggregate. It is more commonly observed with solid microcarriers as cells tend to grow within the structure of macroporous carriers. However, if due to oxygen transfer limitations cells could only grow on the external surface of the carriers then it is conceivable that bridging would occur.

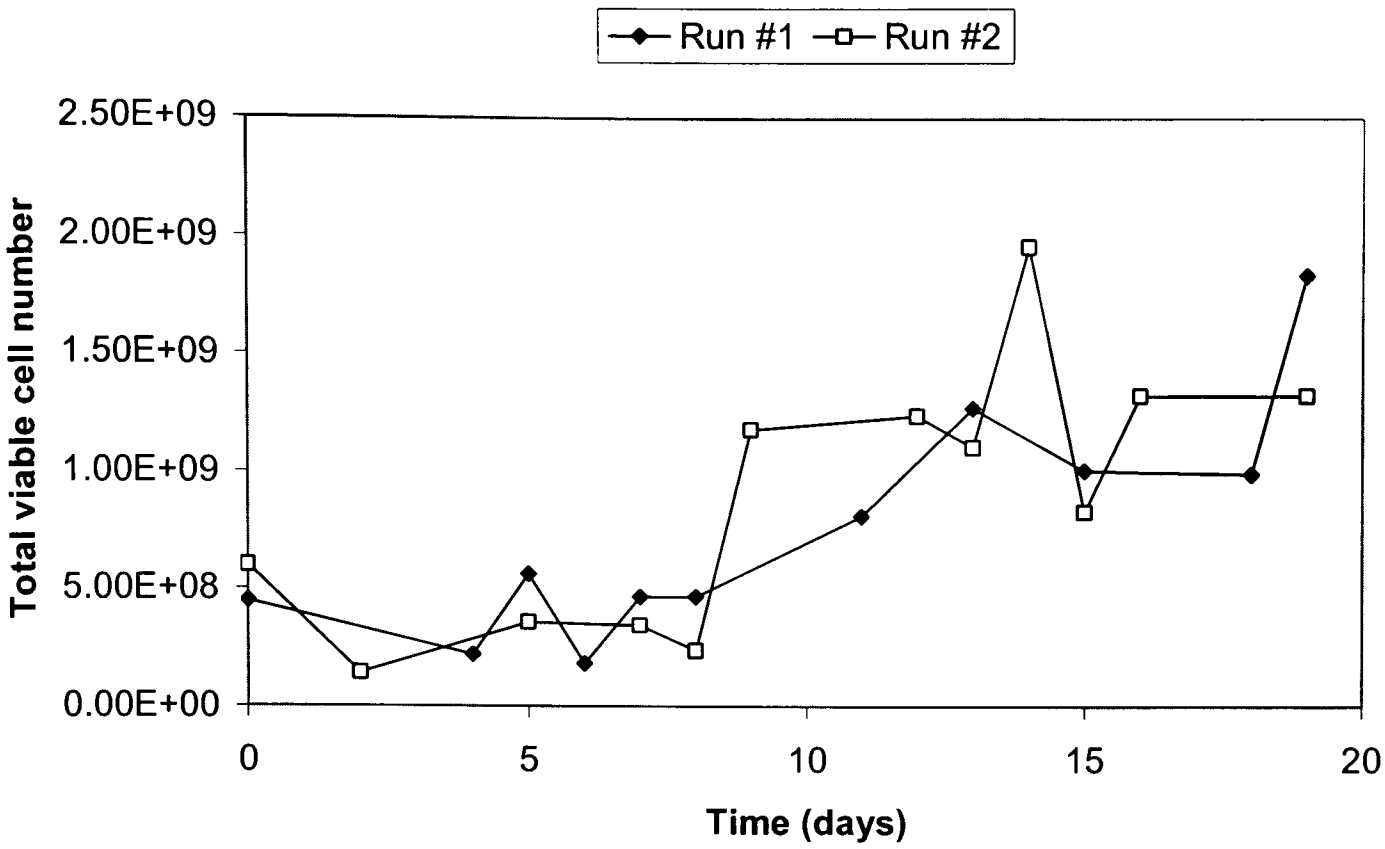
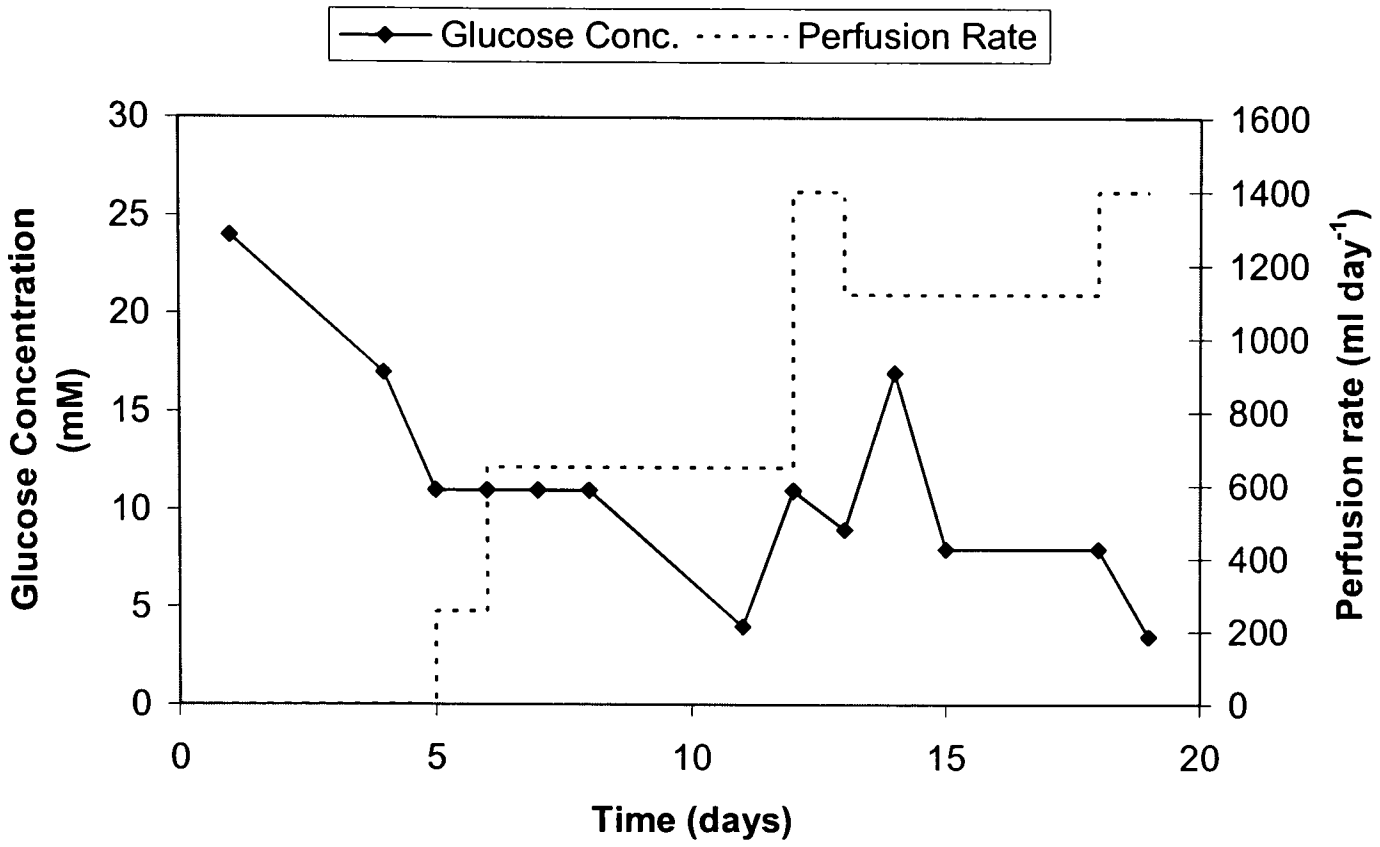


Figure 4.3.5.7

The viable cell number of a perfusion culture of TEFLYRD/83 cells in a fluidised bed bioreactor. Cell numbers were estimated from the GUR. The GUR was calculated from the change in glucose concentration and the perfusion rate. The culture set points were temperature 37°C; DO_2 50%; pH 7.0; fluidisation 100%. The initial impeller speed was 350rpm. During the course of the experiments this was increased to maintain 100% fluidisation.

A



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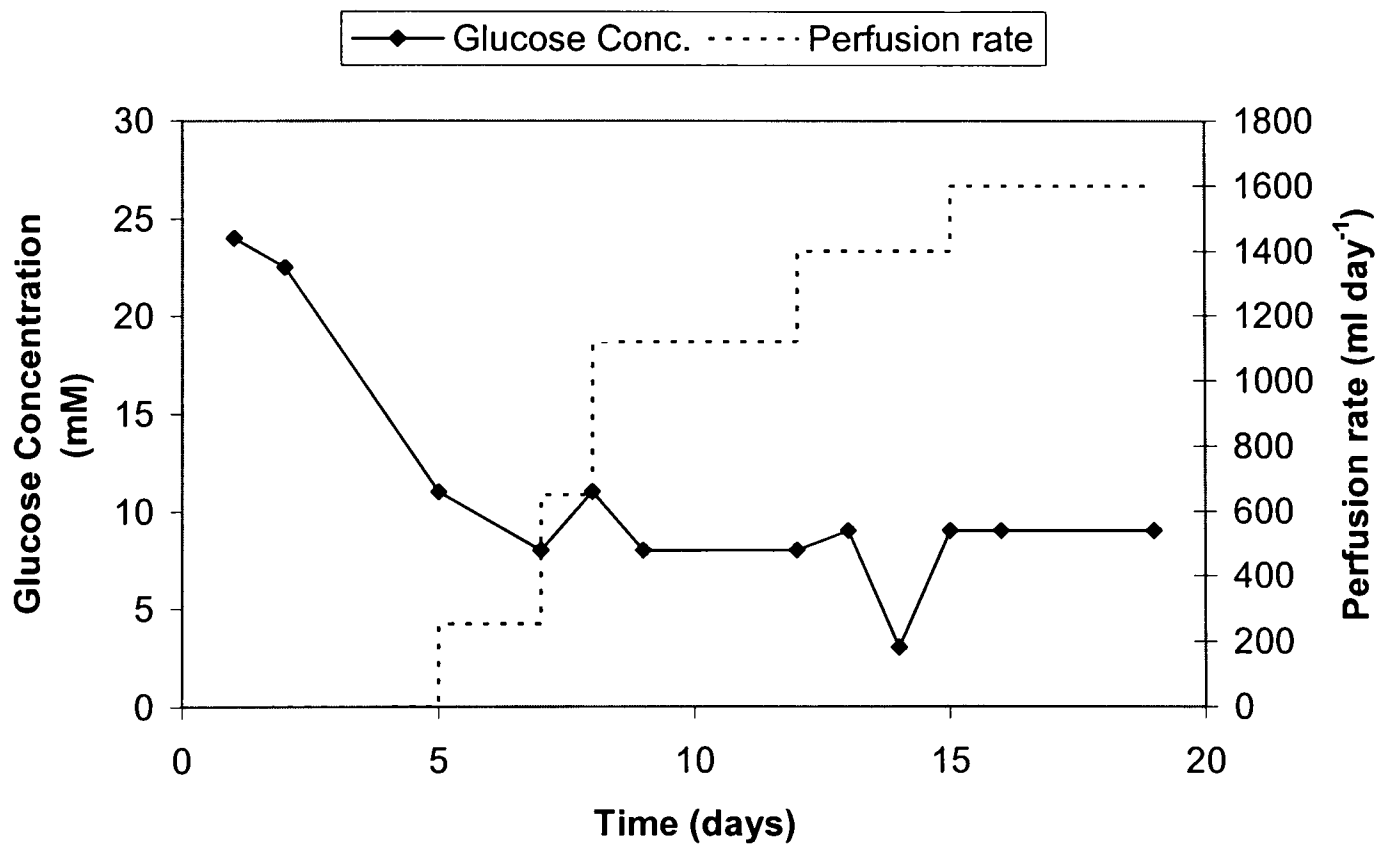

Figure 4.3.5.8

Residual glucose concentration and perfusion rate in a perfusion culture of TEFLYRD/83 cells cultured in a fluidised bed bioreactor. A = run #1, B = run #2. The perfusion rate was adjusted according to the residual glucose concentration.

4.3.6 Retrovirus production in perfusion cultures

On the basis of the cell numbers it was expected that the highest virus titres would be obtained from the fluidised bed bioreactor, followed by the packed bed bioreactor, the fixed bed bioreactor and the lowest titres being from the stirred tank bioreactor. In practice this was found to be true with the exception that higher titres were found in the fixed bed compared to the packed bed bioreactor as can be seen from the data presented in Table 4.3.6.1. No significant amounts of virus particles were detected from the stirred tank bioreactor. It was also presumed that the virus titres would increase over the course of the experiments as the cell number increased, as was the case. This is illustrated for the packed bed bioreactor, the fixed bed bioreactor and the fluidised bed bioreactor in Figure 4.3.6.1, Figure 4.3.6.2, and Figure 4.3.6.3, respectively. This reiterates the fact that virus titre is a function of cell number and high titre vector systems must be able to support high cell numbers.

A direct comparison of vector concentrations between different production systems is complicated due to variations in bed fraction, total working volume and the time of culture. Thus, a more dependable parameter is the specific production rate, assuming that this is constant during the course of the experiment. The highest specific production rate was in the fixed bed bioreactor, which had the lowest cell density and the smallest bed fraction. This system may, therefore, have the greatest potential for effective vector production. The system could easily be intensified by doubling the bed fraction, giving a ratio of carriers:volume equal to that of the fluidised bed bioreactor. Its performance could be further improved by increasing the cell density through optimising the culture parameters.

The increased specific production rate in the fixed bed bioreactor was believed to be due to two factors. In the first instance the specific production rate in the packed bed

bioreactor was thought to be a direct result of the fluid flow rate through the vessel. As mentioned in 4.3.2, fluid flow through a packed bed is a function of bead geometry and flow rate. Effective transport of nutrients to cells and products from the cells is dependent on the level of intraparticle flow. In semi-continuous cultures a relatively high flow rate was used to recirculate medium through the vessel and this resulted in high virus titres. In open perfusion cultures the flow rate was much lower and this may have caused virus particles to get trapped within the immobilisation matrices instead of being transported into the effluent stream. Similarly, the high recirculation rate in the fixed bed bioreactor between the conditioning vessel and the fixed bed would have led to good intraparticle flow, enabling virus particles to be efficiently transported into the supernatant.

The differences in specific production rate between the fixed bed bioreactor and the fluidised bed bioreactor can be attributed to differences in specific growth rates. Through simple kinetic analysis, product formation can be shown to be connected with specific growth rate (Cruz *et al.* 2000). The growth rate in the fixed bed bioreactor and the fluidised bed bioreactor were estimated to be 1.40 day^{-1} and 1.09 day^{-1}, respectively. This accounts for the improved production rate in the fixed bed. The lower growth rate observed in the fluidised bed is due to oxygen limitations as discussed in 4.3.5.

While the specific production rate is of more scientific value for comparison of production systems, bioreactor productivity is of more industrial importance as this parameter serves as the basis for scale-up studies and calculating production plant size (Merten *et al.* 2001). From this viewpoint, the fluidised bed bioreactor was the most favourable system as it was capable of producing virus titres up to 1 x 10^7LTU ml^{-1} (see Figure 4.3.6.3). This is a 11-fold higher than the fixed bed bioreactor. The fluidised bed bioreactor has the added advantage that scale-up is relatively straight

forward compared to packed/fixed bed bioreactors, making it more commercially attractive.

Table 4.3.6.1

Cell growth and retrovirus production parameters in perfusion culture at 37°C in various production systems.

	Stirred tank bioreactor	**Packed bed bioreactor**	**Fixed bed bioreactor**	**Fluidised bed bioreactor**
Bed fraction (bead volume/total volume)	0.1	0.625	0.075	0.15
Initial cell number (x 10^6 cells)	67.1 ± 32.9	20.65 ± 7.05	20.7 ± 13.54	524.5 ± 76
Maximum cell number (x 10^8 cells)	-	2.565 ± 1.55	0.490 ± 0.23	15.8 ± 2.5
Maximum cell density (cells ml^{-1} μcarrier)	-	5.13 x 10^6	3.26 x 10^6	5.26 x 10^6
Maximum virus titre (LTU ml^{-1} day^{-1})	-	8.1 x 10^4	8.9 x 10^5	1 x 10^7
Specific virus production rate (LTU $cell^{-1}$ day^{-1})	-	0.0028	0.237	0.0045

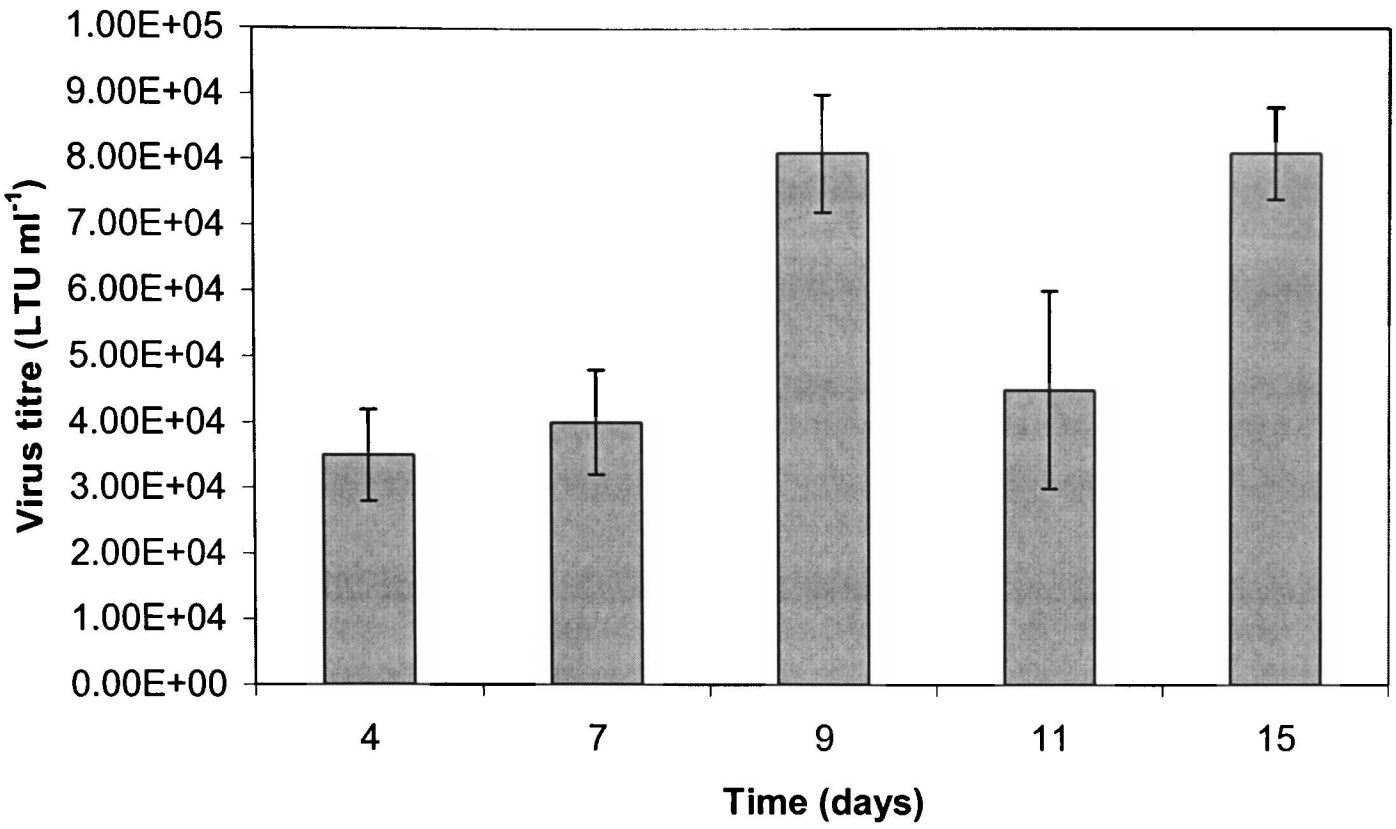

Figure 4.3.6.1

Mean daily virus titres for an open perfusion culture of TEFLYRD/83 cells cultured in a packed bed bioreactor with internal aeration. The supernatant was collected immediately downstream of the culture compartment. The infectious virus titre was measured with the LacZ titration assay. Error bars represent observed range, n = 2.

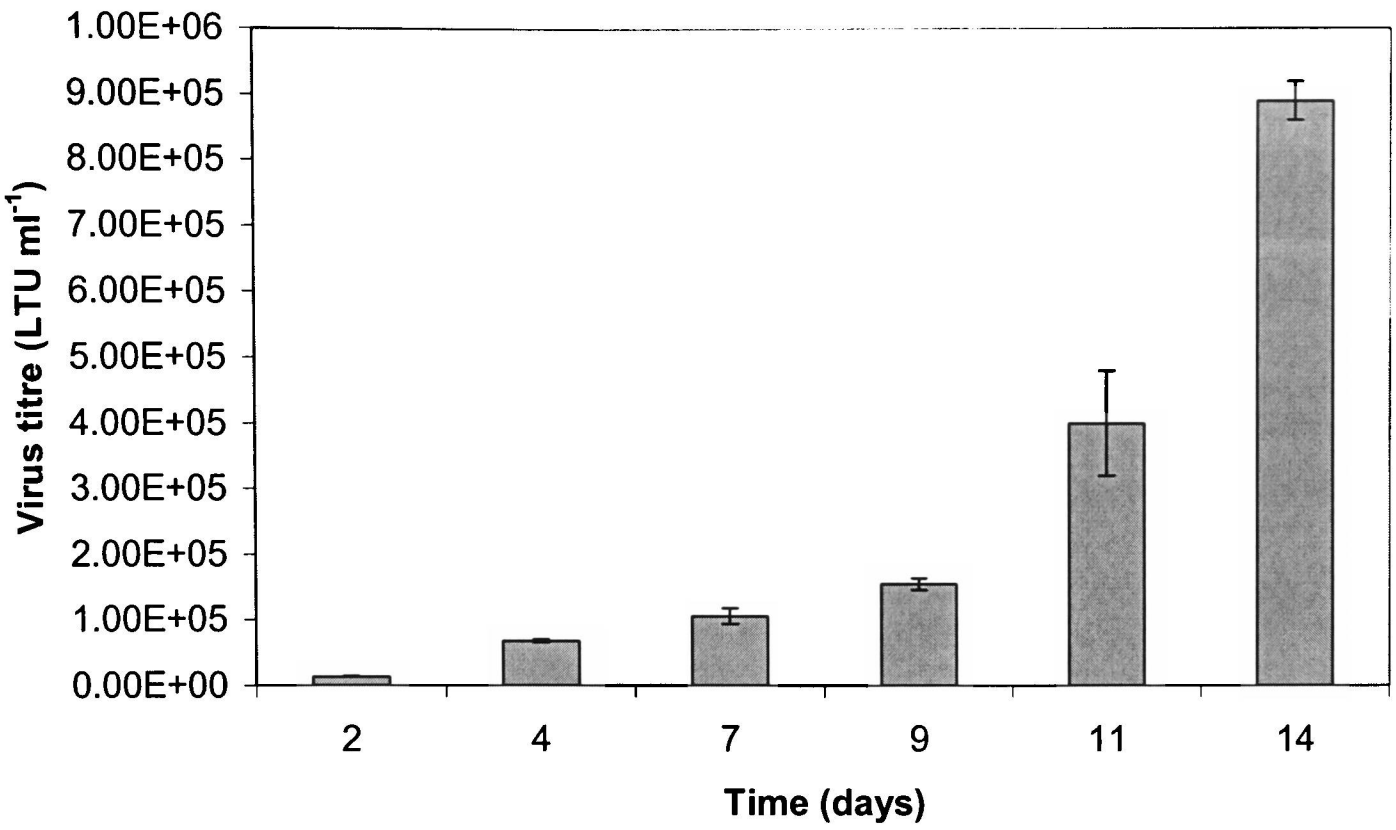

Figure 4.3.6.2

Mean daily virus titres for a perfusion culture of TEFLYRD/83 cells cultured in a fixed bed bioreactor with continuous recirculation of medium between the fixed bed compartment and the conditioning vessel. The supernatant was collected from the conditioning vessel. The infectious virus titre was measured with the LacZ titration assay. Error bars represent observed range, n = 2.

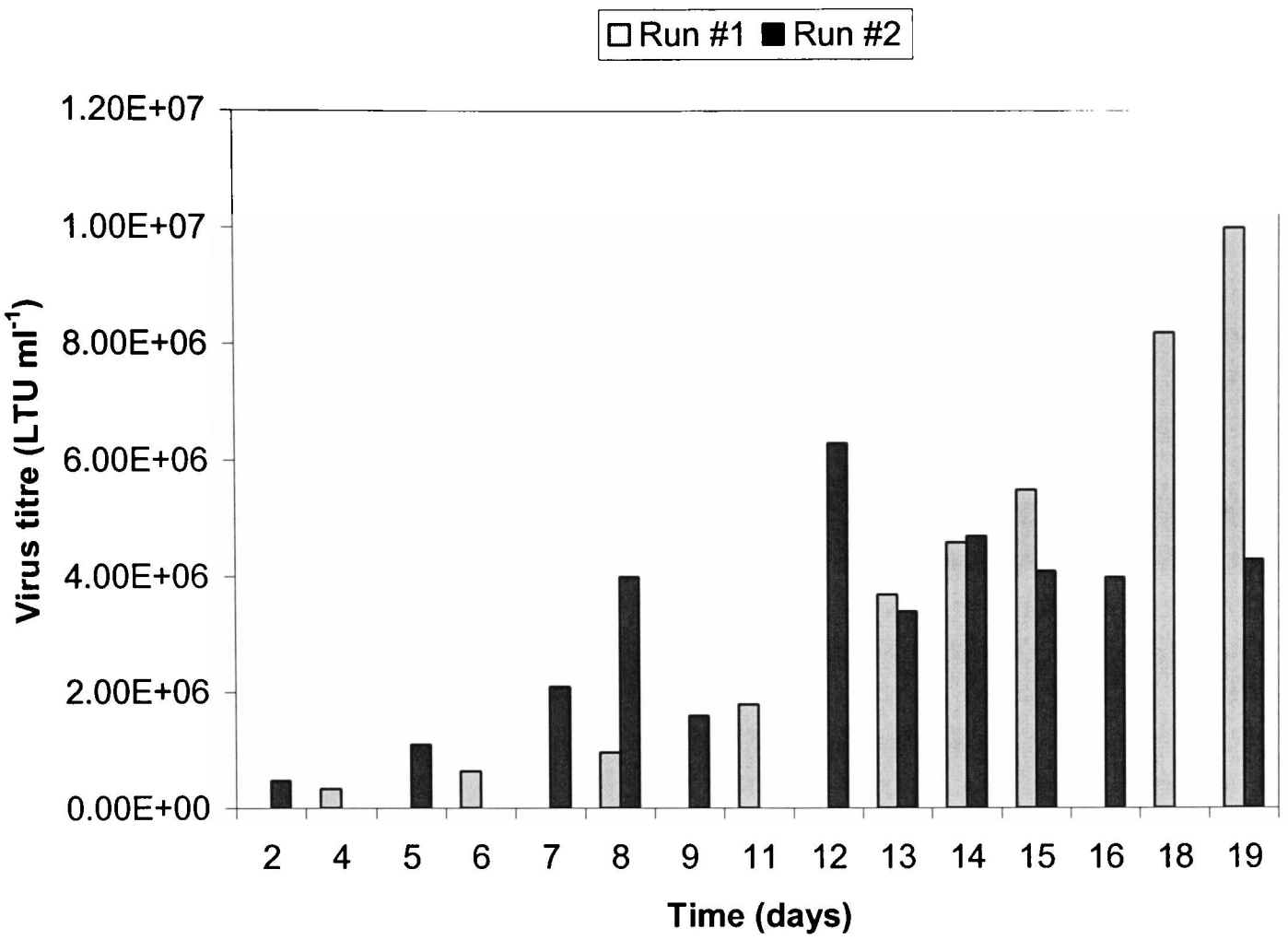

Figure 4.3.6.3

Mean daily virus titres for a perfusion culture of TEFLYRD/83 cells cultured in a fluidised bed bioreactor. The infectious virus titre was measured with the LacZ titration assay.

4.4 Conclusions

A major concern in the production of retrovirus vector stocks is the ability to produce infectious particles efficiently and economically. At the present time clinical grade retrovirus vectors are produced in roller bottle cultures. These are beset with process limitations such as an inability to control important cultural parameters such as pH and dissolved oxygen and scale-up relies on an increase in unit number. Due to these limitations we have evaluated several alternative systems for the production of retrovirus particles.

In the first instance, semi-continuous culture systems, with a production phase at 32°C, were compared to roller bottle culture. Of the three alternative systems that were tested none were able to produce the same volumetric productivities found in the roller bottle, which also had the highest specific production rate. However, the packed bed reactor with internal aeration offers an acceptable alternative. This vessel is still in the developmental stage but through effective reactor design and improvement it could provide a suitable means for virus production. It was proposed that production in this system and in the spinner basket reactor could be enhanced by implementing a continuous perfusion mode instead of semi-continuous feeding.

It has been suggested that perfusion culture at 37°C may be a more efficient means of virus production, with active virus particles being removed from the high temperature compartment and transported to a low temperature harvest vessel, than semi-continuous culture at 32°C. Therefore, four perfusion culture systems were evaluated for long-term virus production. To allow a meaningful comparison to be made the specific production rate was determined based on the final cell number and virus titre. The highest specific productivity was found in the fixed bed reactor and this was attributed to its specific growth rate and the intraparticle convective flow through the

bed of microcarriers. It was hypothesised that its overall productivity could be improved by increasing the ratio of carriers to medium and by improving the cell density by optimisation of the culture conditions. The highest reactor production was observed in the fluidised bed bioreactor. It was thought that this would be the industrial choice of system due to its overall performance and the ease of scale-up.

It was not possible to make a direct comparison between the virus titres in semi-continuous and perfusion cultures. This was due to the different methods used to measure the particles. RT-PCR, used to measure titres in semi-continuous cultures, is a rapid and reliable method. However, the results it produces are based on relative amounts of the fluorescence of the reporter dye. Quantitation is only possible by comparing results to a relative calibration curve. The virus titres for perfusion cultures were measured by the LacZ titration assay. A major drawback of this method, as with any biological assay, is the inherent variability in the number of LacZ transforming units. The ability of virus particles to successfully infect a target cell is dependent on a number of parameters, including the metabolic state of the target cells, target cell density, ratio of target cells to vector particles, concentration of the vector stock, infection medium, exposure time of target cells to vector and the type of target cells (McTaggart and Al Rubeai 2000). Therefore, the virus titres in this study can not be taken as absolute values but as an indication of an increase or decrease in virus number. Comparison between culture systems was possible as virus titres were measured with the same assay and therefore under identical conditions.

CHAPTER 5: INFLUENCE OF SERUM CONCENTRATION ON CELL GROWTH AND RETROVIRUS PRODUCTION AND DECAY KINETICS

5.1 Introduction

To optimize a bioreaction process for retrovirus production, the biological potential of the cell must be optimally exploited and the virus stability maximized. Previous reports on process optimization have looked at the effect of environmental conditions, especially temperature, on virus production (Kaptein *et al.* 1997; Le Doux *et al.* 1999; McTaggart and Al Rubeai 2000), bioreaction mode (Cruz *et al.* 2000) and different bioreactor systems (Merten *et al.* 2001). Previous studies on the effects of serum on retrovirus production have been varied. This may be the result of different cell lines being used in each of the studies. It has been known for almost a decade that product production kinetics depend on both cell line and cell culture method (Leelavatcharamas *et al.* 1994). It has been demonstrated that serum has a negative dose dependent effect on retrovirus production in the FLYRD18 cell line (Gerin *et al.* 1999a; Gerin *et al.* 1999b; McTaggart and Al Rubeai 2000). McTaggart and Al-Rubeai (2000) reported an 11-fold increase in virus titre in cultures containing 1% FCS compared to 5% FCS. The negative effect of serum was determined to be at the virus assembly/maturation stage and not at the extra-cellular stage. This suggested a possible role of protease inhibitors present in serum. In this chapter, the effect of serum concentration on cell growth, virus stability and virus production has been examined. The ability of cells to grow under serum free conditions has also been assessed.

5.2 *Materials and Methods*

5.2.1 Measurement of retrovirus decay

Packaging cells were grown to confluence in 850cm^2 roller bottles at 37°C in DMEM + 10% FCS. The culture medium was removed and replaced with 50ml of fresh medium. After 24 hours the supernatant containing retrovirus particles was harvested and centrifuged at 1500 rpm for 10 minutes to remove any cell debris. The supernatant was then filtered through a 0.22μm syringe filter to ensure sterility. The samples were centrifuged over night (~18 hours) at a speed of 6000g at 4°C to separate the virus particles. The supernatant was removed and discarded and the pellet was resuspended in 5ml DMEM supplemented with either 1% or 10% FCS in sterile bijoux tubes and incubated at 37°C. Samples were taken from the tubes at regular intervals and frozen in liquid N_2 for later analysis by the LacZ virus titration assay.

5.2.2 Measurement of cell growth and retrovirus production in batch culture

Retrovirus producing cells, TEFLYRD/83, were inoculated at a density of ~6 x10^4 cells ml^{-1} medium in several 25cm^2 T-flasks containing 10ml DMEM, supplemented with 10%, 5%, 2.5% and 1% FCS and incubated at 37°C in a 5% CO_2 atmosphere. At regular intervals, the medium from duplicate flasks of each serum concentration was harvested and samples were stored in liquid N_2 for later analysis by the LacZ virus titration assay and the glucose and lactate concentrations were measured as described in chapter 2. After the medium was removed the cells were trypsinised and counted under a microscope using the trypan blue exclusion test and a haemocytometer. The number of cells per flask was assumed to be constant and equal to the mean number of cells per flask observed for each serum concentration.

5.2.3 Long-term monolayer culture and virus production

TEFLYRD/83 human packaging cells were seeded into several 25cm^2 T-flasks at a density of 10^6 cells per flask. 4.5ml of culture medium supplemented with 2.5% or 10% FCS was added to equal numbers of flasks and the cells were grown to confluence over 3 days at 37°C, 5% CO_2. Following this the medium was replaced with fresh medium and the volume was reduced to 1.5ml. Subsequent medium changes were performed at daily intervals. The medium volume was increased as required to prevent glucose limitation. Glucose concentration was measured daily using a "Gluco-Trend" glucose test meter (Roche, France) and virus samples were collected three times per week for later titration. The cell number per flask could only be determined at the end of the experiment by standard trypsinisation methods and counting the number of viable cells using the trypan blue exclusion test. Intermediate cell numbers were estimated from the daily glucose consumption rate.

5.2.4 Retrovirus production in a packed bed bioreactor

Packed bed reactors have previously been shown to be an effective method for the production of retroviral vectors (Kang *et al.* 2000; McTaggart and Al Rubeai 2000; Merten *et al.* 2001). The packed bed used in this study is described in chapter 4, section 4.2.1. The vessel was packed with 5g (50ml) Fibra-Cel™ discs, which were prepared as described in chapter 2.

The bed was inoculated with ~1.7 x 10^7 cells suspended in fresh medium. After 2 hours >90% of cells had attached to the carriers and medium recirculation was started with a medium reservoir of 200ml. The medium reservoir was changed after 4 days and daily thereafter to prevent substrate limitation. Supernatant samples were taken at regular intervals for virus titre and glucose measurements. The cell number was

estimated at the end of the experiment by an MTT assay (see chapter 2). Intermediate cell numbers were estimated from daily glucose consumption rate as described by (Rodrigues *et al.* 1999).

5.2.5 Adaptation to serum-free media

VP-SFM

To determine the ability of cells to grow in a commercially available serum-free, protein-free medium, adaptation to VP-SFM (Gibco Life Technologies, UK) was performed. Cells that had been cultivated in standard culture medium (i.e. DMEM + 10% FCS) were inoculated into T75 culture flasks at a concentration of 10^5 cells ml^{-1}, with 20ml of medium containing VP-SFM and standard culture medium in a ratio of 1:1. Cells were sub-cultured after 3-4 days and the specific growth rate was calculated. When the specific growth rate was >0.35 day^{-1} the concentration of VP-SFM was increased by changing the ratio to 2:1, 4:1, 9:1, 19:1, and 39:1 before cells were grown in 100% VP-SFM. At each stage of the dilution cells were sub-cultured every 3-4 days. A cell bank of cells was prepared as described in chapter 2.

SF-DMEM:F12

Serum-free DMEM:F12 medium was prepared by adding the ingredients listed in Table 5.2.1 to 990ml of double distilled water. The medium was sterilised by filtration through a 0.22μm filter.

Unadapted cells were grown to confluence in a T75 flask with standard culture medium. The medium was removed and replaced with 20ml of SF-DMEM:F12 medium. After 3-4 days cells were passaged and the viability, cell density and specific growth rate were determined. Once cell viability remained above 85% for two

consecutive passages a batch culture was initiated to assess the virus production, as described in 5.2.2.

Table 5.2.1

Ingredients and their concentrations used to prepare SF-DMEM:F12

Ingredient	Supplier	Concentration
DMEM:F12	Gibco Life Technologies	7.88g L^{-1}
$NaHCO_3$		1.125g L^{-1}
ITS-S	Gibco Life Technologies	10ml L^{-1}
0.27% BSA	Sigma	2.7g L^{-1}
0.05% Pluronic F-68	Sigma	0.5g L^{-1}

5.2.6 Kinetic analysis

Previous studies have shown that the production of retrovirus vectors can be described by a mathematical model including differential equations relating to cell growth, virus production and virus decay (Cruz et al. 2000; Le Doux *et al.* 1999; Merten *et al.* 2001). Therefore, the following set of equations can be applied. Cell growth is described by:

$$\frac{dx_T}{dt} = \mu x_v \qquad (1)$$

The specific growth rate is the most suitable parameter for measuring cell growth in given culture conditions (Bailey and Ollis 1986). As growth proceeds it becomes limited by cell or substrate concentration. Limitation of growth and production can generally be regarded as being of two types, stoichiometric and kinetic. A stoichiometric limitation refers to the limitation on product yield, either cell mass or

retrovirus particle production, caused by the complete utilization of a certain nutrient (substrate). A kinetic limitation implies the lessening of a rate, either the growth rate or the production rate, due to a reduction in the amount of the kinetically limiting substrate. Therefore, according to the Monod model

$$\mu = \frac{\mu_{\max} S_0}{(K_S + S_0)} \tag{2}$$

Where μ_{max} is the maximum specific growth rate of the cell line used, S_0 is the initial substrate concentration and K_S is the substrate concentration at half μ_{max}. As cell growth is exponential, the above equation can be integrated and the specific growth rate, μ for each culture can be calculated using the expression:

$$x_t = x_0 \exp(\mu t) \tag{3}$$

where x_t is the cell concentration at time t, x_0 is the initial cell concentration, and t is the time period to the end of the exponential phase.

Retrovirus vectors are particles not molecules and will therefore decay with time. Their level of stability is dependent on several environmental factors including temperature, pH, and serum (Higashikawa and Chang 2001). The rate of decay is proportional to the concentration of active virus and the change in concentration of active virus with time can be described by

$$V^A = V_0^A \exp(-k_d t) \tag{4}$$

where V^A is the concentration of active virus at time t, V_0^A is the initial concentration of active virus and k_d is the virus decay rate constant (Le Doux *et al.* 1999).

The packaging cells were designed to constantly produce retroviral vectors. Assuming that virus production is directly proportional to the concentration of viable cells the concentration of viruses as a function of time can be given by:

$$\frac{dV^T}{dt} = \alpha\mu x + \beta x \tag{5}$$

Where V^T is the total concentration of virus particles in the culture medium, α and β are production parameters characteristic of the cell line and culture conditions, x is the viable cell density, and t is the time. In batch cultures, virus production and virus decay occur simultaneously. Therefore, the concentration of active particles is a function of both production and decay rates:

$$\frac{dV^A}{dt} = \alpha\mu x + \beta x - k_d.V^A \tag{6}$$

The virus decay rate was assumed to be constant and the virus concentration at $t = 0$ was given as 0. By simple rearrangement of equation 6, the specific production rate, k_p, can be calculated and is described as:

$$k_p = \alpha\mu + \beta \tag{7}$$

By definition, the specific production rate (virus $cell^{-1}$ $hour^{-1}$) is therefore associated with specific growth rate.

5.3 Results and discussion

5.3.1 Retrovirus decay

Incubation of retroviral vectors at 37°C in medium supplemented with 10% or 1% FCS showed an exponential decrease in infectious virus particles with time. The decay rate was determined by fitting an exponential curve to the experimental data. For medium supplemented with 1% FCS this was calculated as 0.15 $hour^{-1}$ with a corresponding virus half-life of 4.6 hours. In medium supplemented with 10% FCS the decay rate was 0.114 $hour^{-1}$ which equates to a virus half-life of 6 hours as seen in Figure 5.3.1.1.

Extra-cellular virus particle stability has been shown to decrease with increasing concentrations of human or mouse serum. This is due to the presence of certain sugars on their envelope which activate complement via natural antibodies present in the serum (Takeuchi *et al.* 1996). The packaging cell line and the envelope proteins have been established as the factors that determine which sugars are present (Takeuchi *et al.* 1994). The increased stability of virus particles with an increase in foetal calf serum concentration may be due to two mechanisms. Firstly, the packaging cell line used and the envelope protein used for the vector do not activate complement in FCS. This validates the use of this production system for the generation of clinical grade vectors. Secondly, FCS contains particular components that can enhance the stability of virus particles. Previous reports using packaging cells derived from HT1080 cells, expressing vectors carrying the RD114 envelope protein have reported that FCS concentration has no affect on virus stability (Gerin *et al.* 1999a; McTaggart and Al Rubeai 2000). While this confirms that complement is not activated in FCS, it also demonstrates that the increased stability conveyed by FCS in these experiments is dependent on the packaging cell line, not the envelope protein.

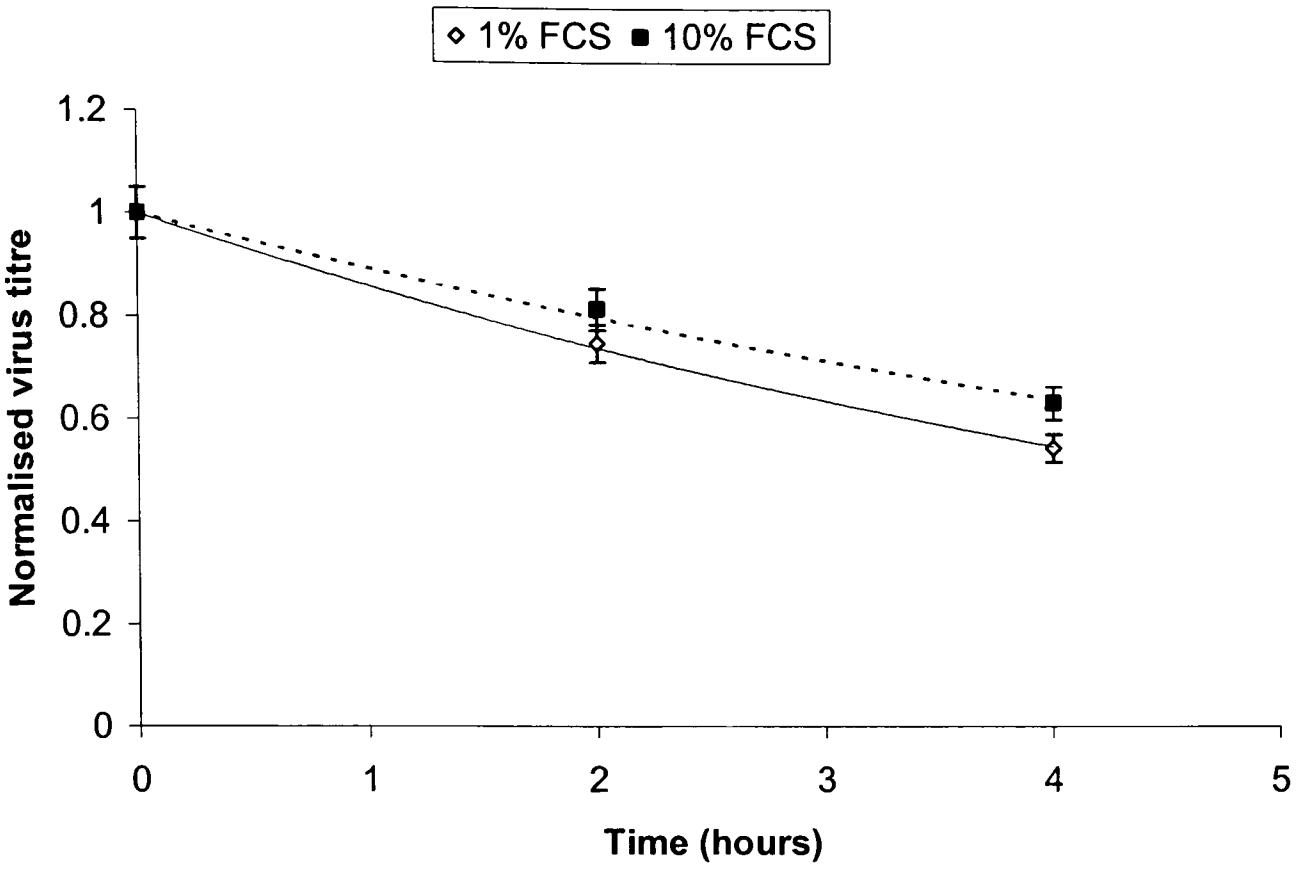

Figure 5.3.1.1

The rate of virus decay in medium supplemented with 10% FCS and 1% FCS at 37°C. Virus particles were produced in roller bottle cultures at 37°C. The supernatant was harvested after 24 hours and the virus was filtered and then concentrated using low speed centrifugation (6000g for ~18 hours). The supernatant was discarded and the pellet resuspended in DMEM supplemented with 1% or 10% (v/v) FCS. The virus suspensions were incubated at 37°C and 5% CO_2. Samples were taken at regular intervals and stored in liquid nitrogen. The amount of infectious particles remaining in the medium was measured using the LacZ titration assay after completion of the experiment. Error bars represent the 95% confidence interval.

5.3.2 Cell growth and virus production in batch culture

The growth of TEFLYRD/83 cells in batch culture at various initial serum concentrations is shown in Figure 5.3.2.1. The maximum cell number achieved was in the range 8.24 x 10^5 cells ml^{-1} to 8.73 x 10^5 cells ml^{-1} at 5% FCS and 2.5% FCS, respectively. The specific growth rate was calculated by fitting the experimental data from the exponential growth phase (between 24 and 144 hours) to equation (3) and the values are presented in Table 5.3.1. There was no significant difference over the range of serum concentrations examined, which shows that serum does not have a kinetic limitation on cell growth. However, the rate of cell death was greater in cultures containing less serum. The reduction in viable cell density, between 192 and 240 hours, was 64%, 41%, 21% and 14% for cultures supplemented with 1%, 2.5%, 5% and 10% FCS, respectively. This supports the hypothesis that serum starvation is an inducer of cell death of a part of the cell population (Kessler *et al.* 1997). Hence, serum exerts a stoichiometric limitation on a cell population.

The residual concentrations of glucose and lactate were measured and are presented in Figure 5.3.2.2. Glucose utilization was essentially linear for all concentrations of serum up to the late exponential phase (144 hours). The rate of consumption was highest in 10% FCS and lowest in 1% FCS. No significant difference was seen between 5% FCS and 2.5% FCS. With the exception of cultures with 1% FCS, the glucose was completely exhausted after 8 days. In cultures containing 1% FCS the growth was limited after 6 days. This corresponds with a glucose concentration of 9.9mMol l^{-1}, suggesting that growth was limited by a factor other than glucose. The production of lactate was consistent with glucose utilisation.

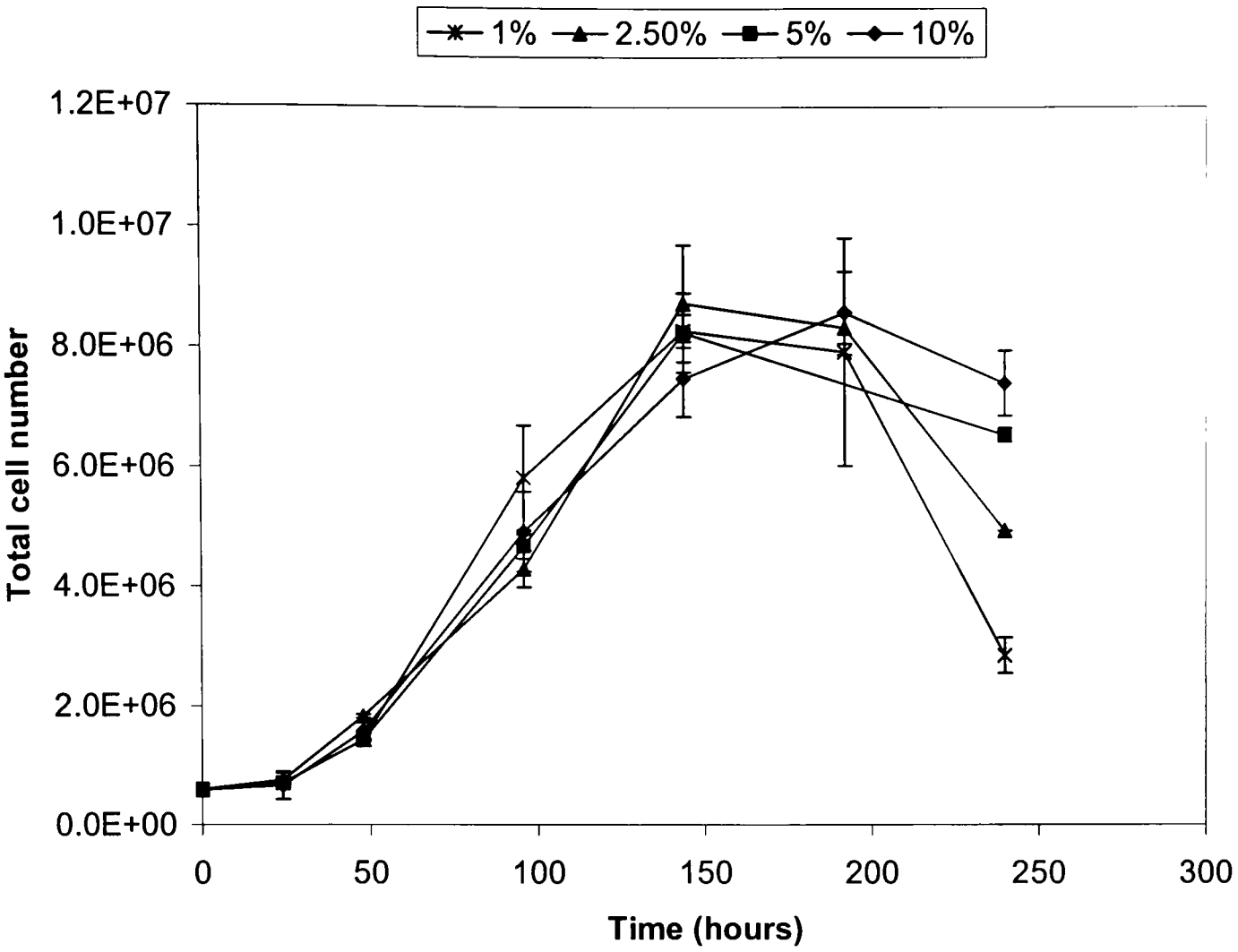

Figure 5.3.2.1

Mean viable cell number during a batch culture of TEFLYRD/83 cells in monolayer at various serum concentrations. At each time point duplicate flasks were sacrificed and the cells were counted after trypsinisation. Cells numbers were assumed to be constant under identical culture conditions. Error bars represent observed range, n = 2.

Table 5.3.1

Cell growth and retrovirus production parameters for batch cultures of TEFLYRD/83 at different initial serum concentrations.

FCS concentration (%)	Specific growth rate, μ ($hour^{-1}$)	Max viable cell density (x 10^5 cells ml^{-1})	Production rate constant, k_p (virus $cell^{-1}$ $hour^{-1}$)
1	0.0204 ± 0.0009	8.27 ± 0.27	0.122
2.5	0.0195 ± 0.0004	8.73 ± 0.97	0.126
5	0.0199 ± 0.0005	8.24 ± 0.66	0.098
10	0.0194 ± 0.0009	8.57 ± 0.69	0.049

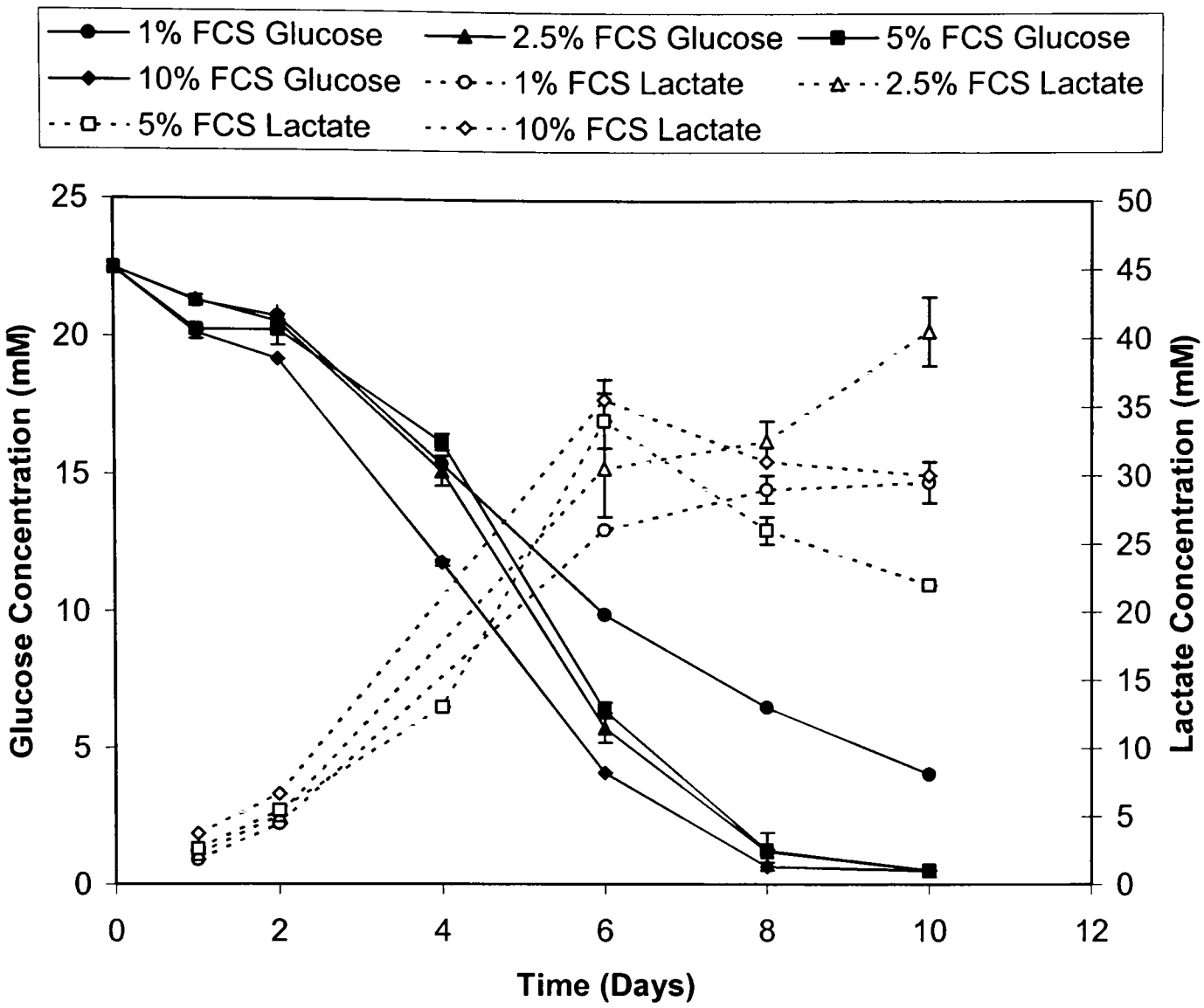

Figure 5.3.2.2

Mean glucose and lactate concentrations in a batch culture of TEFLYRD/83 cells in monolayer culture at various serum concentrations. Duplicate flasks were sacrificed at regular intervals and the residual glucose and lactate concentrations were measured using the GLUCOTREND® glucose test kit and the biolyser rapid analysis system, respectively. Glucose consumption and lactate production were assumed to be constant for all cultures under identical conditions. Error bars represent observed range, n = 2.

The mean daily virus titre was assessed using the LacZ titration assay, and the results are shown in Figure 5.3.2.3. The data presented in Figure 5.3.2.1 and Figure 5.3.2.3 was fitted to equations 6 and 7 in order to determine the specific production rate, k_p, during the exponential phase (see Table 5.3.1). The highest specific production rate was observed in 2.5% FCS, while the lowest was seen with 10% FCS. Hence, there is a direct, negative dose dependent relationship between serum concentration and virus production, as previously reported (Gerin *et al.* 1999a; Gerin *et al.* 1999b; McTaggart and Al Rubeai 2000).

The negative effect of serum on virus production has been attributed to the presence of protease inhibitors (Gerin *et al.* 1999a). During virus replication, the *env* gene is synthesised as a polyprotein that is cleaved by a cellular protease to yield the gp70 (surface protein, SU) and the p15E (transmembrane protein, TM)(Morgan 1995). The presence of protease inhibitors in serum causes a deficiency of env proteins, which has an adverse effect on virus assembly. Therefore, the reduction of serum concentration also reduces the presence of protease inhibitors and enables more active virus particles to be produced. The increase in specific production rate was proportional to the initial serum concentration between 1% FCS and 10% FCS. Therefore, the change in specific production rate with serum concentration could be described as:

$$k_p = c' \exp(-c'' S_0) \tag{8}$$

Where S_0 is the initial serum concentration and c' and c'' are experimentally determined constants. For TEFLYRD/83 cells grown in monolayer batch culture, these were 0.1529 and 0.1082, respectively ($r^2 = 0.95$).

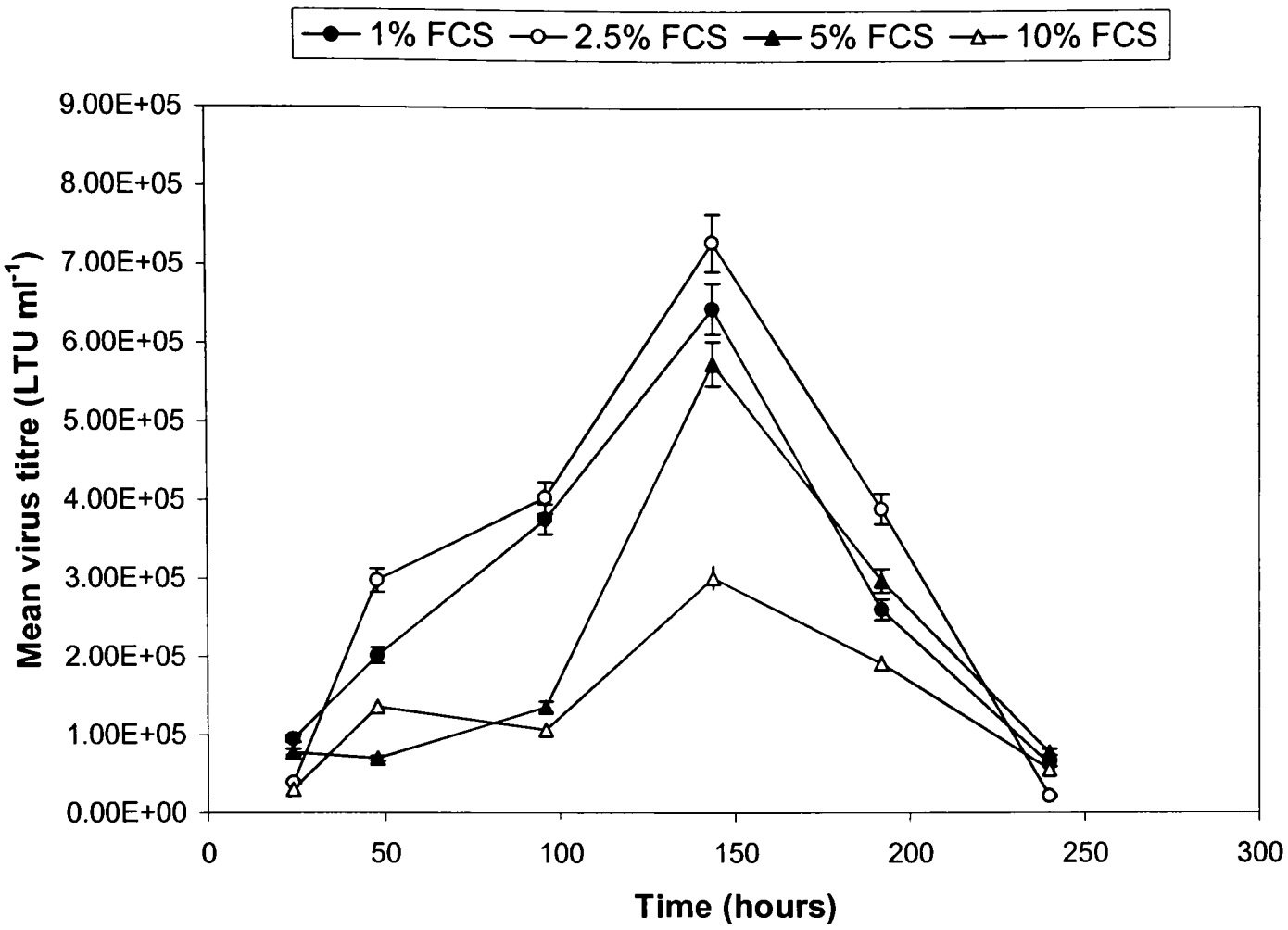

Figure 5.3.2.3

Mean virus titre in a batch culture of TEFLYRD/83 cells in monolayer culture at various serum concentrations. Duplicate flasks were sacrificed at regular intervals and 1ml of supernatant from each flask was stored in liquid nitrogen. The virus titre was measured after completion of the experiment using the LacZ titration assay. Error bars represent the 95% confidence interval, n = 2.

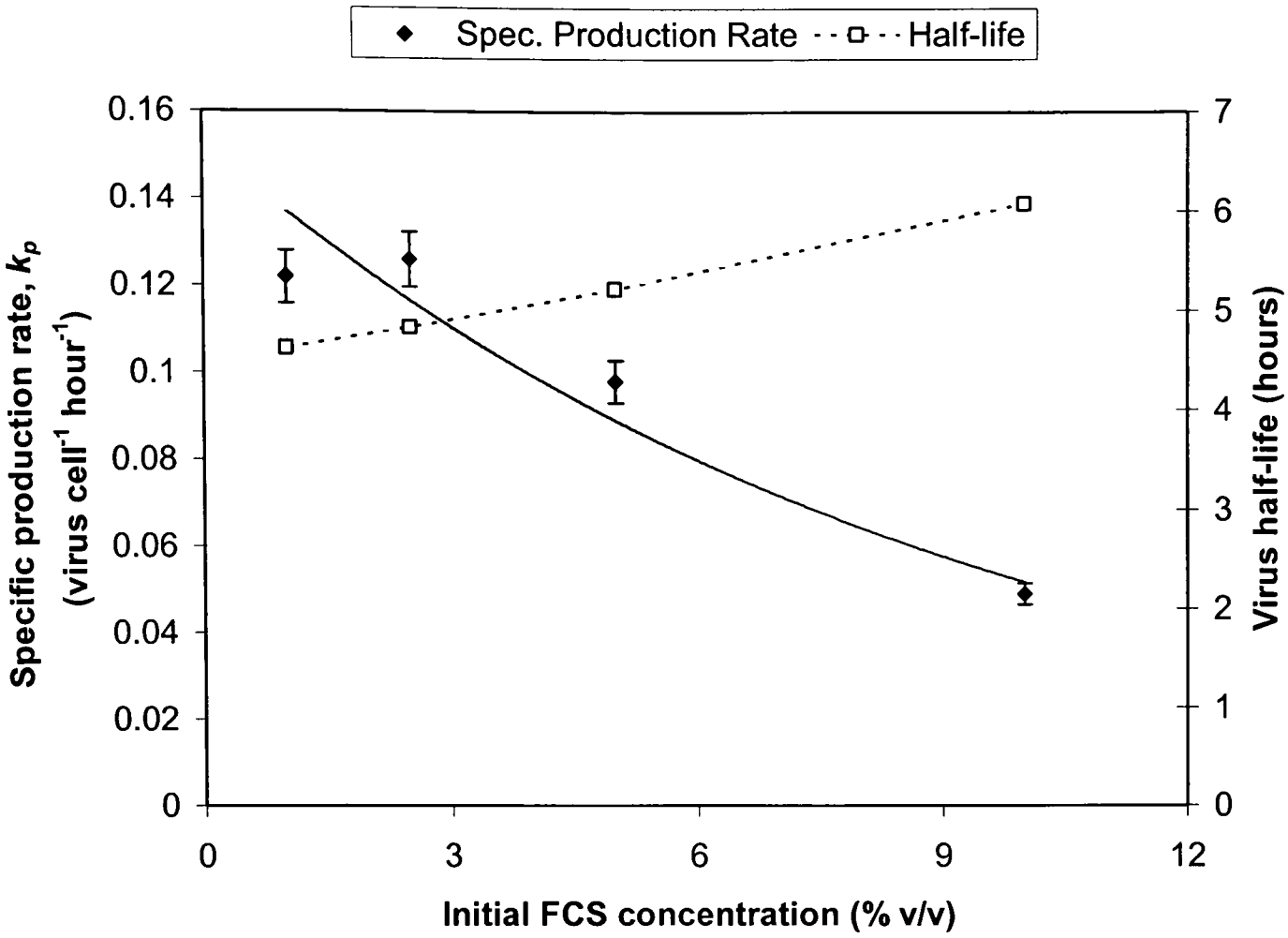

Figure 5.3.2.4

Specific production rate and half-life for OB83 retrovirus vectors produced from TEFLYRD/83 cells grown in monolayer cultures at different serum concentrations. The virus half life was calculated determining the gradient of an exponential curve fitted to the results shown in Figure 5.3.1.1. The specific production rate was calculated by fitting equations 6 and 7 to the experimental data presented in Figure 5.3.2.3. Error bars represent 95% confidence interval.

The highest virus titres were obtained in medium supplemented with 2.5% FCS. This is due to the combination of the specific production rate and the virus decay rate. The virus production rate and the virus half-life were plotted against initial serum concentration, shown in Figure 5.3.2.4. The point at which the lines cross indicates the optimal serum concentration for virus production and stability. Therefore, culture medium supplemented with 2.5% (v/v) FCS offers the most desirable environment for virus production. In addition, the reduction in serum proteins allows more efficient and simplified downstream processing of vector supernatants. A reduction in purification time will further increase the final product yield, making the overall process more economical.

5.3.3 Long-term monolayer culture and virus production

Having determined from batch cultures that culture medium supplemented with 2.5% FCS offered the best environment for virus production, it was necessary to assess the long-term cultivation of cells under reduced serum conditions, in semi-continuous monolayer culture. While the amount of active virus is a function of k_p and k_d, it is also dependent on cell concentration and time, as depicted in equation 6. Therefore, for a constant cell number the virus titre will increase until the specific production rate and decay rate reach equilibrium and a constant virus titre is achieved. By assuming a constant cell density at 10% FCS and 2.5% FCS and applying the constants determined from batch cultures, the optimal residence time was determined to be 24 hours. After 24 hours the increase in virus titre at either serum concentration was inconsequential. For that reason, it was decided to change the culture medium and harvest the virus every 24 hours, once cells had reached confluence.

The cell growth was monitored in monolayer cultures by the glucose uptake rate (GUR). This is a reliable method for the estimation of cell numbers in immobilised

cultures where direct measurement of cell density is not possible (Portner *et al.* 1994; Rodrigues *et al.* 1999). There was no significant difference between the GUR for cells cultured in medium supplemented with 2.5% (v/v) FCS and control cultures, supplemented with 10% (v/v) FCS (Figure 5.3.3.1). The fact that low serum concentration does not affect long term cell density, with daily medium replacement, was confirmed by measuring the final viable cell number, which showed no significant difference. The final viable cell number for 2.5% FCS and 10% FCS was $1.04 \pm 0.03 \times 10^7$ cells and $9.94 \pm 0.77 \times 10^6$ cells, respectively. The final cell number and the virus titres are presented in Figure 5.3.3.2.

An average 42% increase in virus titre was observed in 2.5% FCS cultures compared to 10% FCS over the 21 day period. In batch culture, the average daily increase in virus titre was over 2-fold higher in 2.5% FCS compared to 10% FCS. This indicated that virus production was effected by a parameter other than serum concentration. As all culture conditions were consistent with batch cultures it was suggested that cell growth rate has an effect on virus production. In a previous study on the relationship between cell proliferation, cell cycle and virus production it was proposed that without significant cell proliferation virus particles are not produced (McTaggart and Al Rubeai 2001). During batch culture the specific growth rate was high as cells were in the exponential growth phase. However, once cells have reached confluence the total cell number remains comparatively constant as cell population cannot increase due to a limiting factor such as surface area or substrate exhaustion. Thus, the specific growth rate, μ, is essentially zero. Therefore, the Leudeking and Piret equation for product formation, as described in equation 6, will become:

$$\frac{dV^A}{dt} = \beta x - k_d V^A \qquad (9)$$

And k_p is now equal to β. The significantly lower production rates in semi-continuous culture imply that α is the dominant parameter in cell specific production and cell proliferation is important for achieving high virus titres.

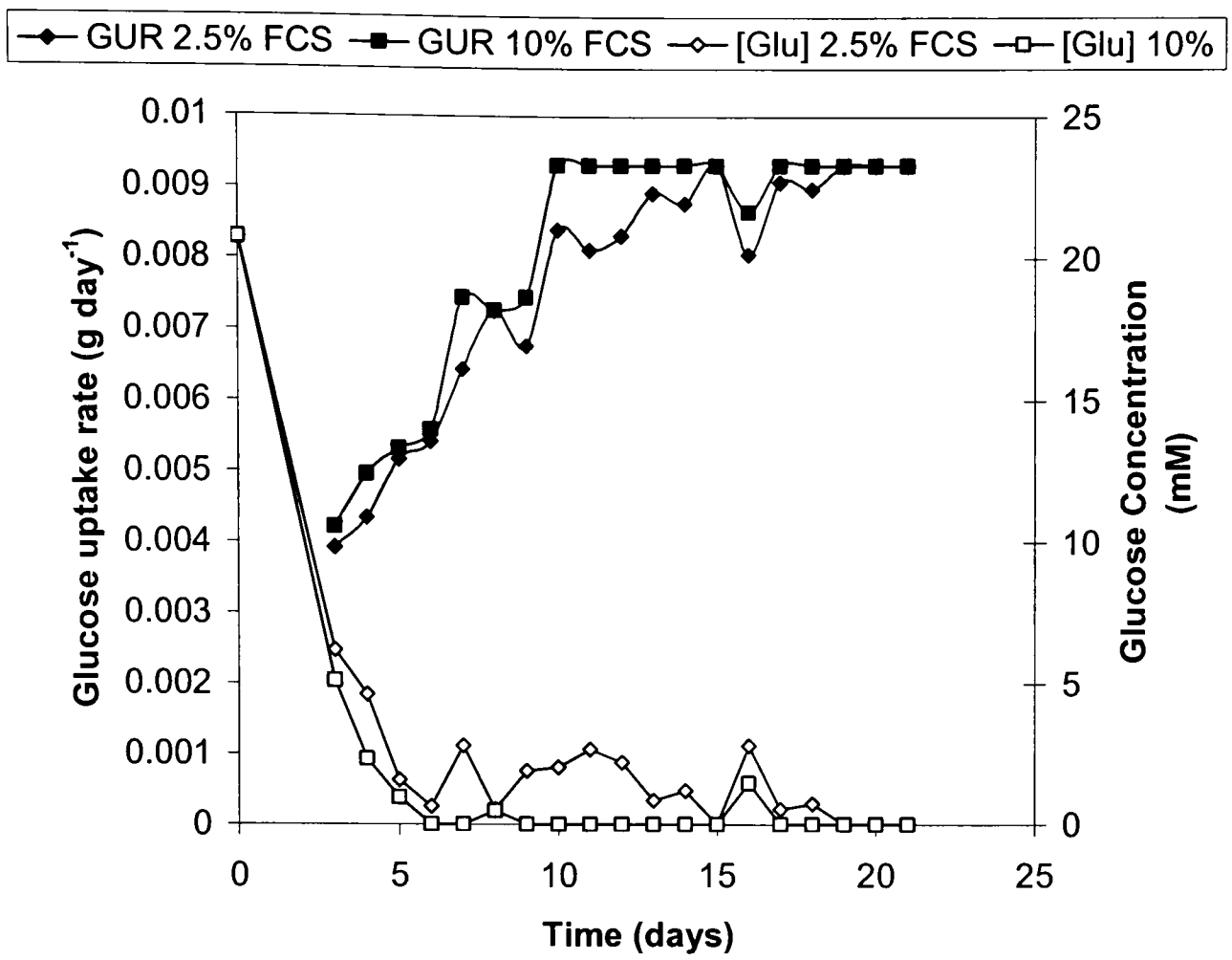

Figure 5.3.3.1

Mean glucose uptake rate (GUR) and residual glucose concentration in a semi-continuous culture of TEFLYRD/83 cells in triplicate monolayer cultures. Medium was supplemented with 2.5% (v/v) FCS and 10% (v/v) FCS.

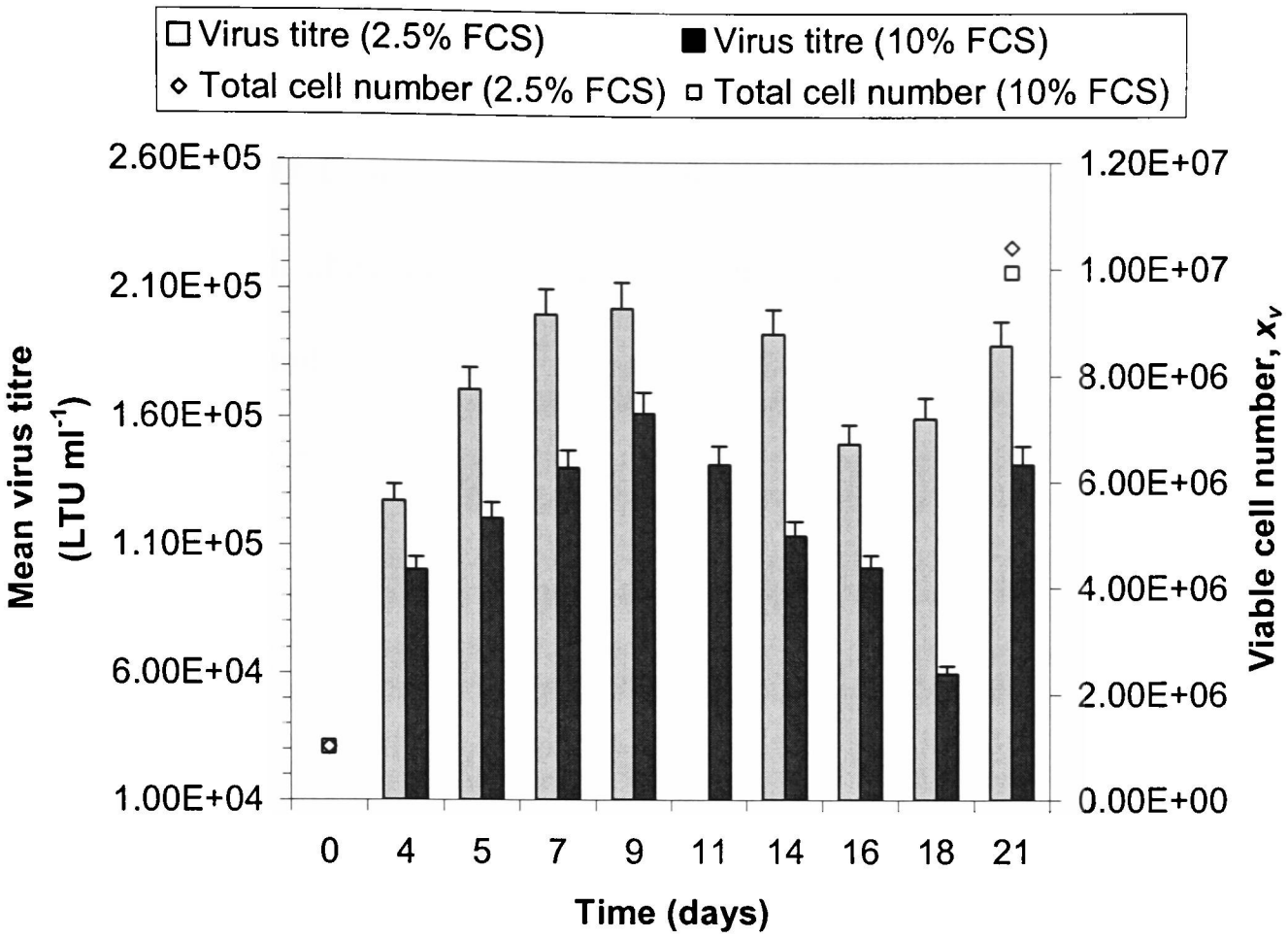

Figure 5.3.3.2

Mean daily virus titre and cell density in a semi-continuous culture ofTEFLYED/83 cells in monolayer containing medium supplemented with 2.5% and 10% FCS. The virus titre was measured from harvested supernatant by the LacZ titration assay. The final cell number was obtained by trypsinising cells and counting under a microscope using a haemocytometer counting chamber. Error bars represent 95% confidence interval, n = 3.

5.3.4 Retrovirus production in a Packed-bed reactor

Although the results from long-term monolayer cultures were not as promising as hoped for, they still showed that a reduction in serum concentration could enhance virus production and appreciably improve the final yield. However, monolayer cultures are undesirable for large-scale production as scale-up relies on multiple flasks or bottles as oppose to a single high volume, high surface area unit process. Packed bed reactors have previously been shown to offer a suitable means of immobilising packaging cells and producing virus vectors (Kang. *et al.* 2000; McTaggart and Al Rubeai 2000; Merten *et al.* 2001).

The cell growth was monitored through the residual glucose concentration and the GUR (Figure 5.3.4.1). The medium was changed after 4 days when glucose began to limit cell growth. At this point the GUR showed no difference between cultures supplemented with 2.5% (v/v) FCS and control cultures (10% (v/v) FCS). After the first medium change the GUR between control cultures and test cultures began to diverge, indicating that cell growth in 2.5% FCS was constrained. Loss of cell viability in immobilised cultures can be due to numerous factors that include nutrient limitation, toxic metabolite accumulation, changes in pH or mass transfer limitations. As the test culture was under identical conditions to the control culture, the decrease in cell growth and premature loss in viability could only be attributed to the reduction in serum concentration. This phenomenon had not been previously observed in monolayer cultures. Serum is essential for certain cell lines to maintain function, growth and attachment. In static monolayer cultures, cell attachment occurs as a function of gravity, as cells have a higher density than the culture medium. Once cells have attached they are not exposed to any hydrodynamic forces that may result in their detachment as the culture medium is motionless. In contrast, culture medium

constantly flows through the packed bed in order to supply the cells with nutrients and remove toxic metabolites. Fluid flow through a packed bed can be regarded as flow through a collection of entangled, tortuitous tubes (Park and Stephanopoulos 1993). A pressure gradient exists that is proportional to fluid velocity and this will exert a friction factor on the external and internal surface of the carriers, as described by the Ergun and Carmen-Kozeny equations (Rhodes 1998). The reduced serum concentration results in a reduction of attachment factors and the effect of the friction factor cause cell detachment and loss of viability. This force is relatively small compared to those found in stirred cultures and detachment is not observed immediately after the start of medium circulation. Therefore, the fluid flow through the packed bed is a key parameter in determining optimal performance. If the fluid flow is too low diffusion gradients will appear resulting in dead zones within the vessel due to mass transfer limitations, which will confine cell number. Conversely, high flow rates will increase the friction factor resulting in cell detachment.

In contrast to monolayer cultures the virus titre was lower at 2.5% FCS compared to 10% FCS, as was expected from the cell density (see Figure 5.3.4.2). The specific production rate was also lower at both serum concentrations when compared to semi-continuous monolayer cultures. At 10% FCS β was calculated to be 0.001 LTU $cell^{-1}$ $hour^{-1}$ in the PBR, compared to 0.005 LTU $cell^{-1}$ $hour^{-1}$ in monolayer, and in 2.5% FCS β was calculate as 0.0001 LTU $cell^{-1}$ $hour^{-1}$ in the PBR compared to 0.008 LTU $cell^{-1}$ $hour^{-1}$in monolayer. This demonstrates that β is not constant and will vary according to the culture conditions. The change in environment, and the difference in serum concentration, almost certainly has an affect on cell function. As a consequence the translational and transcriptional machinery of the cells may be altered, which contribute to reduced virus levels.

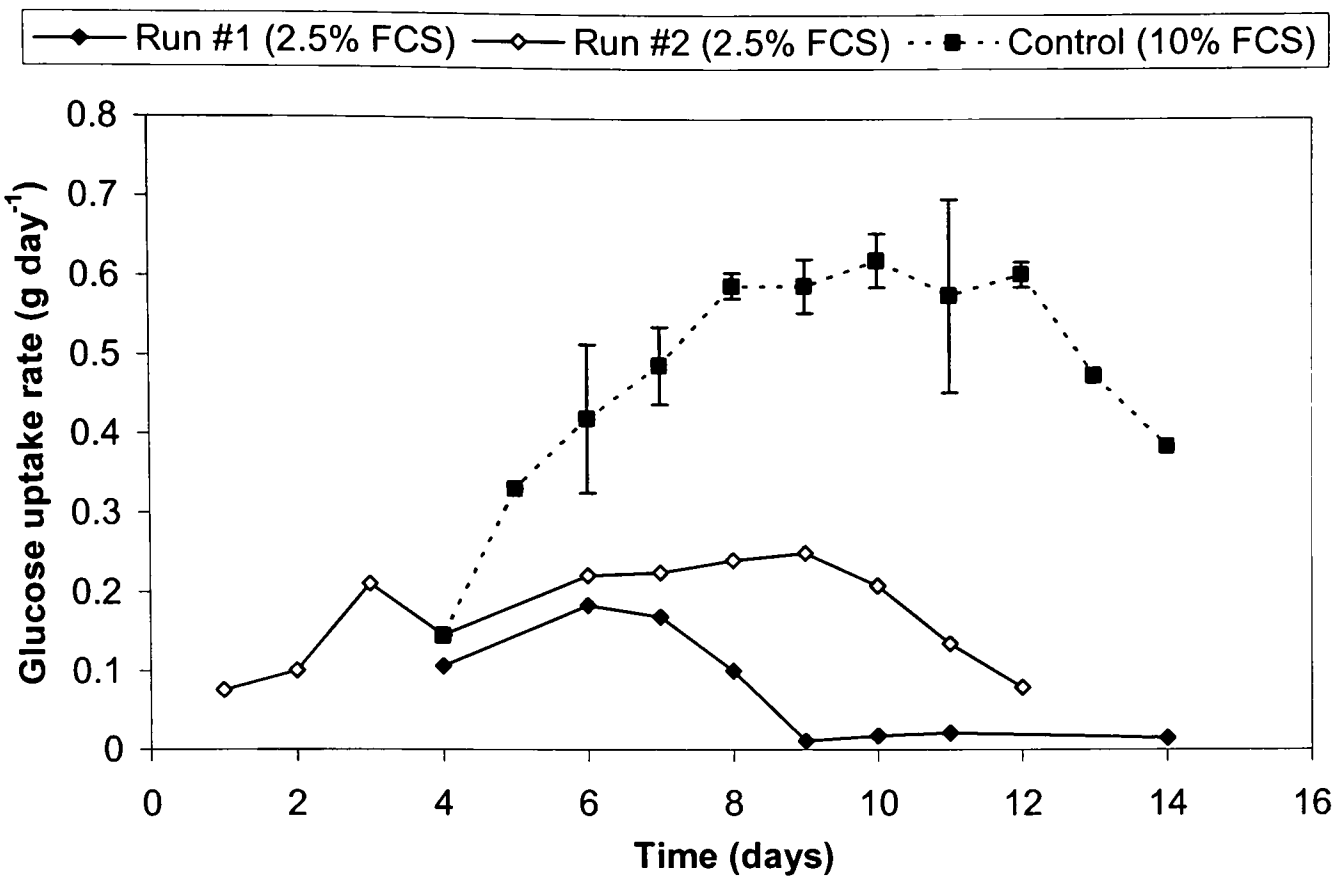

Figure 5.3.4.1

Glucose uptake rate in a semi-continuous culture of TEFLYED/83 cells on Fibra-Cel™ discs in a packed bed bioreactor with medium containing 2.5% or 10% FCS. Medium was changed after 4 days and 6 days, then every subsequent day. Error bars for medium supplemented with 10% FCS represent the observed range, n = 2.

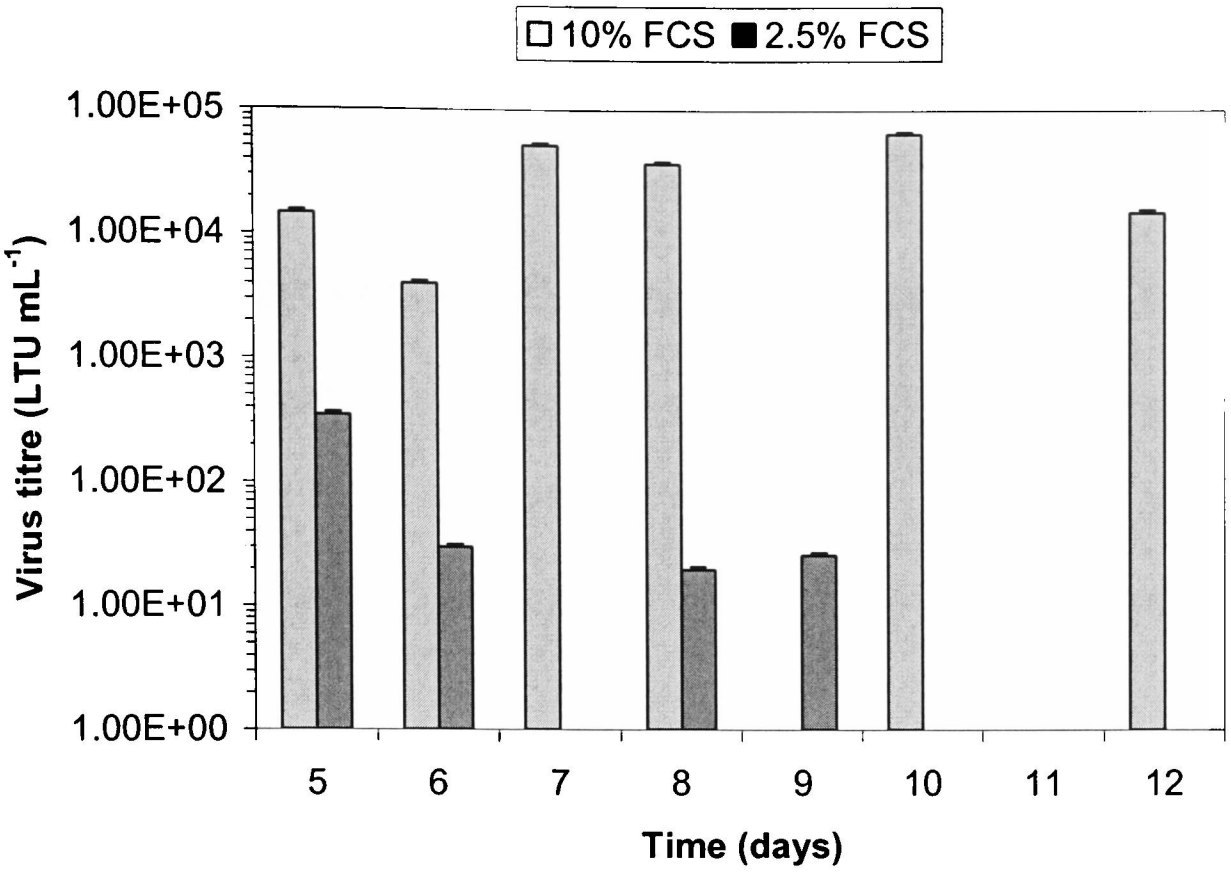

Figure 5.3.4.2

Mean daily virus titres in a semi-continuous culture of TEFLYRD/83 cells on Fibra-Cel™ discs in a packed bed bioreactor with medium supplemented with 2.5% FCS or 10% FCS. The virus titre was measured from harvested supernatant using the LacZ titration assay. Error bars represent 95% confidence interval, n = 2.

5.3.5 Adaptation to serum-free media

VP-SFM

Adaptation of TEFLYRD/83 cells to VP-SFM serum-free, protein-free medium was performed over a period of 38 days. Cells were adapted to grow in medium containing 0.25% FCS with a specific growth rate of 0.25 day^{-1}. The specific growth rates and doubling times at different serum concentrations during the adaptation process are shown in Figure 5.3.5.1.

A cell bank was created before complete removal of serum from the culture medium. However, cells revived from the cell bank lost their ability to grow in medium containing less than 5% FCS, although growth characteristics appeared normal in medium containing 10% FCS. In addition to this, when cells were cultivated in DMEM supplemented with 10% FCS it was observed that virus production was negligible. Therefore, it can be deduced that the freezing solution had an adverse affect on cell function following their adaptation to low-serum medium.

Development of a suitable freezing medium is now required to fulfil the potential of this work. Successful adaptation to serum-free, protein-free medium will offer a wide range of benefits to the production of retrovirus production such as reducing the risk of contamination by foreign viruses, mycoplasma, and bacteria, elimination of protease inhibitors, and simplification of downstream processing. The absence of components of animal origin will also make the production process more attractive to regulating authorities.

Successful adaptation to VP-SFM may also allow cells to be adapted to suspension cultures. Although it is not possible to achieve the same cell densities in suspension culture as with immobilisation methods, it is advantageous with regard to scale-up

and is a method commonly used in industry. This is an important consideration if large-scale production is to be undertaken by a contract manufacturer as their facilities would most likely be designed for suspension cultures.

Another advantage of suspension cultures are that a continuous process could be used. This would allow the removal of cells and virus and by doing so would encourage the division of cells retained in the bioreactor vessel. As virus production has been proved to be linked with cell growth rate it is proposed that this method would enhance the productivity of the system.

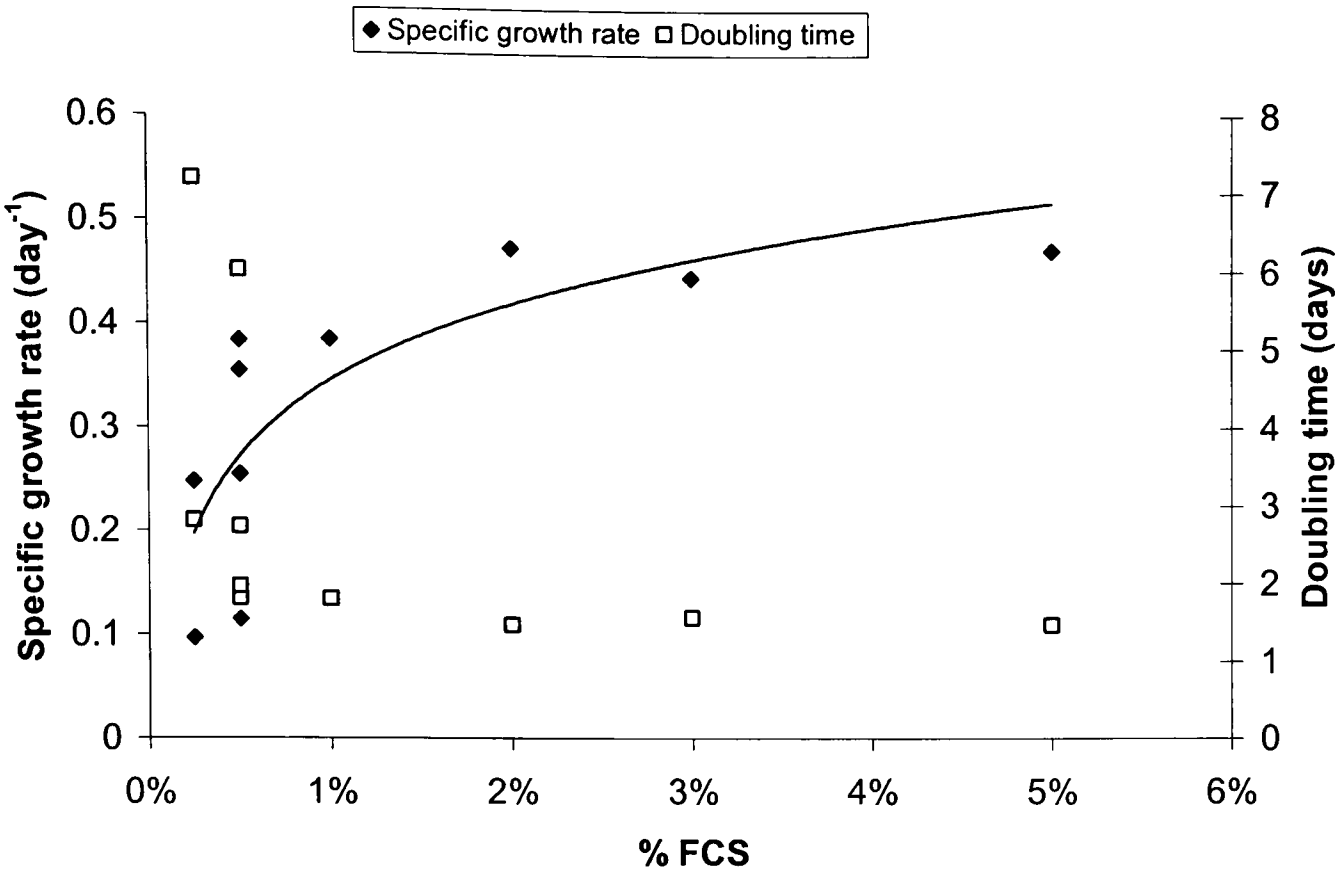

Figure 5.3.5.1

Specific growth rate and doubling time of TEFLYRD/83 cells during adaptation to VP-SFM serum-free medium. Cells were cultivated in $75cm^2$ T-flasks at the respective serum concentrations. They were passaged every 3-4 days and the specific growth rate was calculated using equation 3. The doubling time was determined from the corresponding growth rate.

SF-DMEM:F12

Following the successful survival of cells in SF-DMEM:F12 medium over a period of 21 days, a batch culture was performed to establish the growth characteristics of cells and the virus production in relation to cells cultured in DMEM + 10% FCS. This medium composition has previously been used to grow a variety of cells lines under serum-free conditions including CHO cells (Sakai *et al.* 1999), and hybridoma cells (Murakami *et al.* 1982).

The growth of cells is shown in Figure 5.3.5.2. No increase in cell number was observed in the serum-free medium and after 7 days the viable cell number had fallen to zero. Cell death was ascribed to the absence of serum in the medium as the control culture, supplemented with 10% FCS, displayed typical growth characteristics of the cell line. Serum is a complex medium component that promotes cell replication (Bailey and Ollis 1986). It was anticipated that the addition of albumin and ITS-S would be able to replace this capability. However, in light of these results it is apparent that TEFLYRD/83 cells were unable to replicate in SF-DMEM:F12 medium. The inability of cells to survive in this culture was possibly due to a loss of cell function. During the initial 24 hours of the culture the glucose concentration was dramatically reduced (see Figure 5.3.5.3). A reduction on glucose concentration is usually associated with cell growth but this was not the case. It is more likely that glucose was required by the cells for maintenance energy as a result of being inoculated at a low density into serum-free medium. Prior to this study, cells had been cultivated in serum-containing medium and this was only substituted by serum-free medium when cells were at a high density. After the first day no significant change in glucose concentration was observed suggesting that cell metabolism had completely ceased.

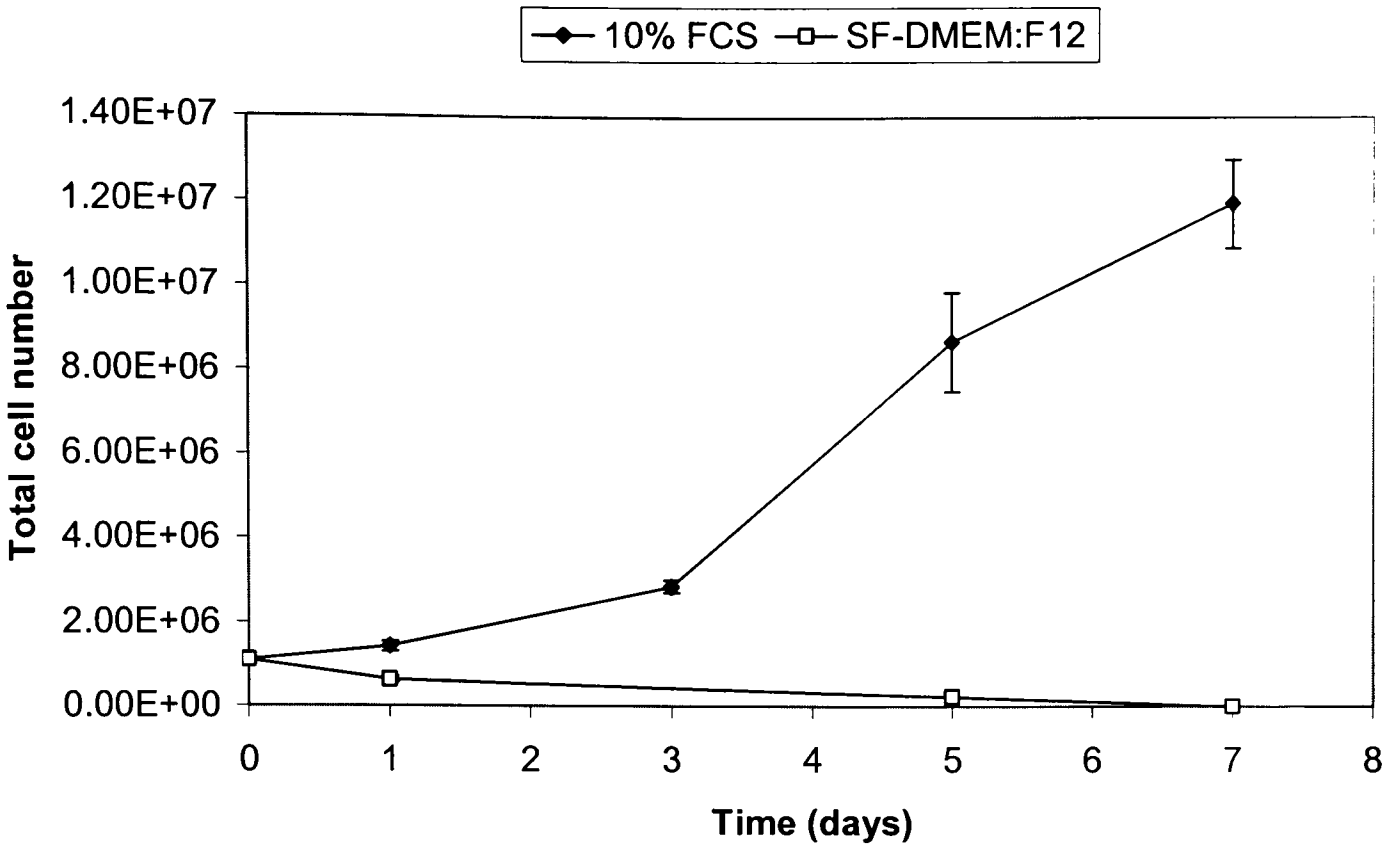

Figure 5.3.5.2

Mean viable cell number in a batch culture of TEFLYRD/83 cells in monolayer culture with DMEM + 10% FCS or SF-DMEM:F12 media. Cells were inoculated at a density of 10^6 cells $flasks^{-1}$ in to several $25cm^2$ T-flasks with 10ml of medium. Duplicate flasks were sacrificed at regular intervals and the cell number was determined by trypsinising cells and counting under a microscope using a haemocytometer. Viability was determined by the trypan blue exclusion test. Error bars represent observed range, n = 2.

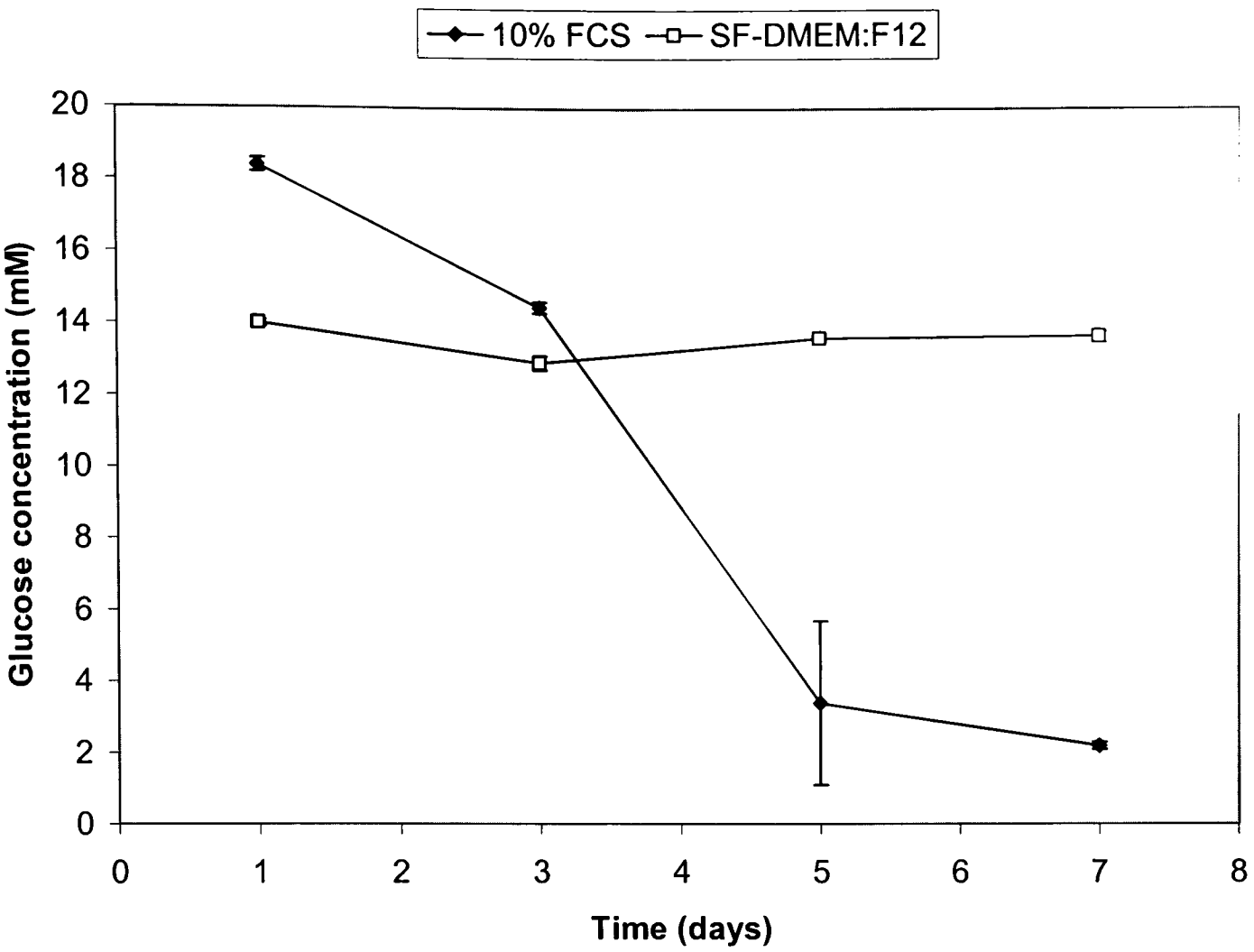

Figure 5.3.5.3

Residual glucose concentration in a batch culture of TEFLYRD/83 cells in monolayer with DMEM + 10% FCS and SF-DMEM:F12. The glucose concentration was measured from the supernatant collected from sacrificed flasks using the GLUCOTREND® glucose test kit. Error bars represent observed range, n = 2.

Despite the diminishing cell numbers and a lack of glucose uptake, the cells retained their ability to produce infectious virus particles, as seen in Figure 5.3.5.4. The titres obtained were lower than in control cultures, as expected from the cell densities. However, this does show that cells were able to maintain genetic functionality under serum-free conditions.

The implications of this study are that cells could be grown in serum containing medium before the serum concentration is gradually reduced to allow production of virus vectors under serum-free conditions. Cells would not necessarily have to be cultivated in high concentrations of serum during the initial stages as it has been demonstrated that growth rate is not affected at initial serum concentrations of 1% in monolayer cultures. This method has been successfully used to increase production of rabies virus in microcarrier cultures (Frazzati-Gallina *et al.* 2001). However, the effect of reducing serum concentration in packed bed bioreactor cultures requires further investigation to determine how cells will respond.

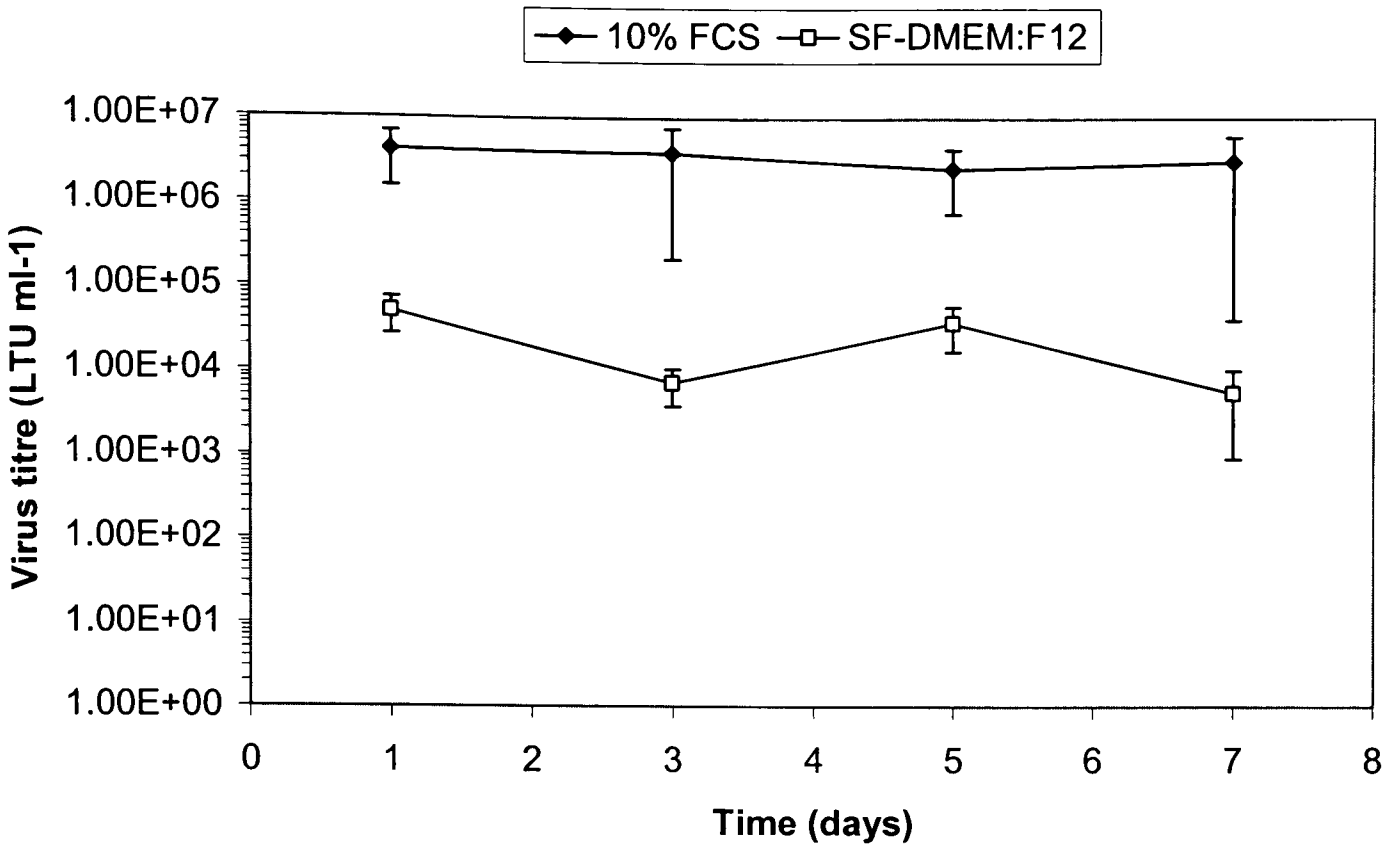

Figure 5.3.5.4

Mean virus titre in a batch culture of TEFLYRD/83 cells in monolayer with DMEM + 10% FCS and SF-DMEM:F12. Flasks were sacrificed at regular intervals for enumeration of cells. 1ml of virus containing supernatant was collected from these flasks and stored in liquid nitrogen. After completion of the experiment the virus titre was analysed using the LacZ titration assay. Error bars represent observed range, n = 2.

5.4 Conclusions

In this work it has been confirmed that serum concentration has a direct negative influence on virus production in human packaging cell lines. The presence of antiproteases in serum inhibits the assembly of virus particles at the intracellular level. However, certain components of serum can increase the stability of extra-cellular virus particles, justifying the use of low concentrations of FCS.

Kinetic analysis shows that two parameters, α and β, influence the specific virus production rate. Of these, α is the dominant parameter and is related to the specific growth rate. Therefore, the specific production rate is higher in batch cultures where the cells are in the exponential growth phase and the growth rate is relatively high, compared to semi-continuous cultures, where the growth rate is essentially zero. β is a weak function of production rate and is influenced by the culture environment and its affect on cell function. This has been proved by the difference in production rate observed between monolayer cultures and the packed bed reactor.

A reduction in initial serum concentration may offer some benefit to the production of retroviral vectors for use in production. However, it is essential that a culture system that allows cells to be kept in a highly proliferative state is used to fully exploit the benefits of the increased virus production rate.

Complete adaptation of cells to grow in serum-free medium has not been achieved. However, promising results have been produced. Successful adaptation to VP-SFM is perfectly feasible if a suitable freezing solution can be developed. This may then lead to the adaptation of cells to grow in suspension. Cells were able to survive and produce virus particles in SF-DMEM:F12, although cell replication was not observed. Therefore, this medium may be useful in developing a process where serum concentration is gradually reduced during the course of the culture.

Chapter 6: General discussion and conclusions

As gene therapy products progress through clinical trials and become closer to being fully licensed products, the need for efficient and economical methods of manufacture grow in ever increasing importance. To see the realisation of such processes a number of key issues have to be overcome. The two major concerns are with the stability of the virus particles and the low productivity of the packaging cell lines. The stability of viruses is affected by a number of external factors. These include temperature, pH of the culture supernatant and the concentration of serum (Higashikawa and Chang 2001; McTaggart and Al Rubeai 2000). While increasing the stability of virus particles can significantly improve virus titres, there is still a need for enhanced virus production. Cells do respond differently to the substrate to which they are attached to, the feeding strategy, the type of bioreactor in which they are grown and the type of medium used to feed them. These factors all have an influence on specific growth rate, death rate and production rate and an understanding of the cellular reaction to all these features is essential for process optimisation. It is hoped that in this study the problems that face the gene therapy industry have been addressed through the alternative systems that have been presented.

6.1 Mode of bioreaction

Batch culture systems are the most popular production system because of their simplicity in design and operation and the fact that they are well used and validated. Any alternative mode of bioreaction has to maintain these characteristics and be economically advantageous (Griffiths 1992). In monolayer culture, batch operations were shown to be very effective for the production of virus particles and it was possible to obtain relatively high virus titres, providing the particles were harvested at

the end of the exponential growth phase. Previous studies have alluded to the fact that a direct correlation exists between the specific virus production rate and the specific growth rate (Cruz *et al.* 2000; McTaggart and Al Rubeai 2001; Merten *et al.* 2001). This was subsequently confirmed through kinetic analysis (see chapter 5). Therefore, it is not surprising that in semi-continuous culture the virus titres were considerably lower than the maximum titre achieved in batch. The advantage of semi-continuous operation is that virus particles can be produced over long cultivation periods and it is envisaged that the accumulated titre will be higher than the equivalent titres achieved from a corresponding number of batch operations over the same time period. It is also proposed that the volume of medium can be reduced from batch cultures as nutrients are only required for multiple 24 hour periods as opposed to 144+ hours in batch. However, in practice the batch cultures remained superior to semi-continuous production. The total number of virus vectors was not higher than the equivalent titres produced in batch and the anticipated advantages in volume of medium used were unfounded.

In semi-continuous cultures the cells are grown to maximum density and then virus containing medium is harvested every day. Cells can survive over extended periods because nutrients are replenished every day and toxic waste products are removed before their presence becomes detrimental. In this system, though, the cell number remains constant, once the maximum density is achieved as dead cells are immediately replaced and the density is limited by either surface area availability or nutrient exhaustion. This has a negative effect on virus production because it is related to specific growth rate. Cells retain their ability to produce infectious particles but their productivity is greatly reduced by the fact that the specific growth rate is essentially zero i.e. there is no change in the cell number.

In order to improve the semi-continuous process the temperature during production was reduced from 37°C to 32°C. At lower temperatures the cell productivity has been shown to decrease. However, the virus stability is a stronger function of temperature and is enhanced at 32°C, resulting in a significant improvement in the virus titre (Le Doux *et al.* 1999; McTaggart and Al Rubeai 2000). When the temperature was reduced in monolayer semi-continuous cultures a marked increase in the virus titre was observed and the total amount of virus produced was far superior to that obtained from batch cultures. Batch culture at 32°C was not deemed to be a viable production method as the decrease in temperature would have a unfavourable effect on cell growth.

The disadvantage of semi-continuous culture is that it is difficult to implement in large-scale processes. The constant removal and replenishment of medium may also increase the risk of contamination. Hence, continuous perfusion cultures at 37°C were examined. At 37°C cell metabolism will be able to operate under optimal conditions and the specific production rate is presumed to be at its highest with regard to cell function. Virus particles are continuously removed from the culture vessel, overcoming the problem of rapid viral inactivation at 37°C. In addition the perfusion rate can be set to meet the nutritional requirements of the cells, resulting in a more efficient feeding strategy. Perfusion cultures can be applied to large-scale processes and their use in existing manufacturing processes demonstrates their regulatory acceptance. Direct comparison of virus titres between semi-continuous and perfusion cultures was not possible due to the different methods used to measure infectious virus particles; RT-PCR was used to measure the virus titre from semi-continuous cultures and the LacZ titration assay was used for perfusion cultures. Perfusion cultures were shown to offer a viable alternative to semi-continuous production in

roller bottles, with virus titres up to 1 x 10^7LTU ml^{-1} being achieved in a fluidised bed reactor.

Due to the relationship between cell growth and virus production an alternative mode of operation may be a chemostat. In this system the cells are not retained in the bioreactor but are continuously removed. The cell number in the reactor remains constant, though, as cells continue to actively divide. This method would maintain cells in a highly proliferative state, whilst maintained a constant high cell density and virus particles would be continuously removed from the high temperature vessel. However, prior to evaluation of this system it is necessary to adapt cells to grow in suspension cultures. This has not been possible to date.

6.2 Selection of a suitable microcarrier

The suitability of a microcarrier as an immobilisation matrix for cultivation of cells can be evaluated with regard to rate of attachment, cell growth and survival and virus production. The carriers studied were chosen on the basis that previous research had demonstrated their suitability for cell propagation and production of a range of biopharmaceutical products in a variety of culture systems. None of the four microcarriers evaluated in this study were solid beads, as this type has an inferior surface area to volume ratio, when compared to macroporous carriers, and cells are exposed to hydrodynamic forces in stirred vessels.

The rate of attachment was rapid for all microcarriers (Cytoline 1, Cytoline 2, Immobasil FS and Fibra-Cel™) with >90% of cells attaching to the microcarriers within 2 hours. In stirred cultures, agitation was started after 24 hours. There was no reduction in cell number after the start of stirring in Cytoline 1 & 2 cultures. This shows a strong cell attachment was achieved or that cells were able to colonise the interior pores of the matrix where they were protected from hydrodynamic forces. In

contrast a significant reduction in cell number was observed in cultures containing Immobasil FS carriers. Despite rapid attachment, a sharp drop in viable cell number after the onset of mixing proves that a weak cell attachment was present or that cells were unable to rapidly proliferate into the porous structure. Cells did recover and were able to grow. However, cells were unable to reach a high cell density or maintain viability over an extended period of time. This shows that this type of carrier is unsuitable for the cultivation of TEFLYRD/83 cells.

Fibra-Cel™ discs were able to sustain cell viability and growth under both static and stirred conditions. In addition to this, a high virus titre was achieved in batch culture.

Successful cell growth on a microcarrier requires that medium be efficiently supplied to the cells. A major problem with macroporous carriers is that nutrients, such as glucose or oxygen, cannot be efficiently transported to cells that colonise the interior of the particle. At the same time metabolic products accumulate and are unable to diffuse into the free supernatant resulting in cell death in the centre of the bead (Yamaji and Fukuda 1992). As the cells in this necrotic core lyse their cellular components are released and will adversely affect the surrounding cell population (Fassnacht *et al.* 1999). Virus particles may also be trapped within the particle, increasing their residence time in the vessel, leading to lower titres. These problems can be overcome by (i) decreasing the size of the particle and thus reducing the distance for the transport of nutrients and products, and (ii) increasing the pore size. Smaller macroporous beads, such as Cytopore, are commercially available. The problem with these microcarriers is that they are difficult to handle, because of their size, and they are only suitable for use in stirred bioreactors. One of the purposes of porous structures is to protect cells from mechanical forces. An increase in pore size would be counter productive as eddies would exist that were smaller than the pores

and the level of protection would be reduced. Fibra-Cel™ discs have partially overcome these drawbacks by modifying the geometry and structure of the carrier. A disc shape allows a short diffusion pathway, allowing efficient transport of nutrients and products to and from the cells. The woven structure offers a macroporous environment that has a large surface area and protects cells from shear stresses. However, these carriers are only suitable for production in packed bed bioreactors. Therefore, these systems must be optimised in order to fully exploit the advantages presented by Fibra-Cel™ discs.

6.3 Choice of bioreactor

It is apparent from this work that the most suitable system for the production of infectious virus particles is either the fixed bed bioreactor or the fluidised bed bioreactor. The use of packed bed bioreactors, incorporating Fibra-Cel™ discs for cell immobilisation, has been previously reported and shown to offer great potential for large-scale processing of vectors (Kang *et al.* 2000; Merten *et al.* 2001). The Cytopilot fluidised bed bioreactor is a novel method for retrovirus production and its appropriateness has not been previously reported.

An important consideration in choosing a production system is its capability for industrial scale manufacture. The fixed bed bioreactor had a higher cell specific production rate but the overall reactor productivity was poorer than the fluidised bed bioreactor, due to a lower cell number. The reactor productivity may be improved by intensifying the culture system, by increasing the bed volume in relation to the conditioning vessel volume, and by optimising the culture conditions to improve the cell density. Another drawback in the use of the fixed bed bioreactor is its scalability. It has been reported that the maximum length of the fixed bed is 15cm. However, this apparent disadvantage can be overcome by the use of a radial flow fixed bed (Pörtner

et al. 1999). This has shown good scale-up potential and a 5 litre fixed bed within a 10 litre glass conditioning vessel can achieve an equivalent cell density to a 100 litre suspension culture. However, unless the overall productivity of the fixed bed bioreactor can be improved, the fluidised bed reactor remains the industrial choice for virus production. Additionally, the fluidised bed bioreactor is favourable because it is a scalable system, with vessels up to 400 litres commercially available.

There are a number of problems associated with the fluidised bed bioreactor. Of greatest significance is the poor cell densities achieved. These have been attributed to oxygen transfer limitations, which prevent the cells from completely utilising the porous structure of the microcarriers. Cell densities between 2×10^7 and 3×10^7 cells ml^{-1} have been reported for this system using CHO cells (Goldman *et al.* 1998; Kong *et al.* 1999; Wang *et al.* 2002), and it is believed that through process development similar cell densities could be achieved with the TEFLYRD/83 cell line. Another drawback observed in the fluidised bed bioreactor was the clumping of microcarriers approximately 18 days into the culture. This led to the termination of the experiment after 19 days, whereas the fixed bed bioreactor was successfully run for 28 days. The clumping of carriers was mainly attributed to the high serum concentration in the medium and it was thought that reducing this would result in improved performance.

The production in either of these systems could be enhanced by modifications in the process. As with semi-continuous cultures the temperature could be reduced to 32°C. Originally the culture temperature was set at 37°C and a low residence time was expected in order to prevent excessive virus deactivation. However, in practice the perfusion rates in these systems was not as high as anticipated and the residence time of the virus particles was greater than 24 hours. Therefore, a significant amount of particles would loose their infectivity before they could be harvested from the vessel.

Hence, a reduction in culture temperature is expected to improve the infectious virus titres. A further consideration is to reduce the serum concentration over the course of the experiment. In the fluidised bed reactor this would prevent microcarrier clumping, as mentioned above, but it would also increase the specific cell production rate (see chapter 5). An initial serum concentration of 10% (v/v) would be required for adequate cell attachment but it is proposed that this could be reduced in a step-wise fashion until the concentration was 1% (v/v). These suggested improvements require thorough validation but it is anticipated that they will be able to enhance the overall productivity of perfusion culture systems.

6.4 Medium development

The standard culture medium used for the production of retrovirus vectors (as stipulated by Oxford BioMedica) is DMEM supplemented with 1% (v/v) non-essential amino acids and 10% (v/v) foetal calf serum. The kinetic analysis of batch cultures has proved that the initial serum concentration has a direct negative effect on the specific production rate. This is due to the presence of protease inhibitors in the serum which hinder the assembly of infectious virus particles. The virus stability was also affected by the serum concentration. The half-life was reduced at lower concentrations suggesting that certain serum proteins are able to enhance the vector stability. The cell specific growth rate showed no significant difference between serum concentrations above 1% (v/v). Therefore, the optimal serum concentration was determined to be 2.5% (v/v). This was verified in semi-continuous culture, although the difference in virus titre between 2.5% and 10% was not as great as in batch culture. This was due to the fact that the specific growth rate in semi-continuous culture was essentially zero, which influenced that specific production rate. The advantages of reduced serum concentrations could not be emulated in packed bed bioreactor cultures.

This is thought to be due to the reduced levels of certain serum components that are required for the attachment of cells to the microcarriers. This problem could be overcome by the addition of attachment enhancing supplements to the medium as a substitute to the serum components. Alternatively, the cells could be grown in an initial concentration of 10% FCS and weaned off this over the duration of the culture, as discussed in the previous section.

Two serum-free media were tested to ascertain their suitability for cell cultivation and virus production. Cells were adapted to grow in VP-SFM supplemented with 0.25% FCS. However, they were unable to retain this capability after a single freeze-thaw cycle. This highlights the need to develop a suitable freezing medium that will permit cells to preserve the ability to proliferate under near serum-free conditions, and hopefully enable a completely serum-free culture to be accomplished.

Cells also demonstrated an ability to survive in SF-DMEM:F12 medium. However, they were unable to proliferate when inoculated into a batch culture. Cell function was retained and virus particles were produced, albeit at lower concentrations, for the duration of the experiment. This may be a suitable alternative medium in semi-continuous cultures or perfusion cultures once cells have reached confluence and virus production has begun.

Modifications in the medium composition are a very important aspect of process design. Traditionally, animal cell culture has used serum as it contains proteins necessary for cell growth, function and attachment. In recent years, though, alternatives have been sought in order to dispose of all components of animal origin due to the safety concerns that have been raised. This is especially true for products used in human therapy. It represents a major source of contamination from viruses, mycoplasma and bacteria and the introduction of pyrogenic contaminants by serum

complicates product recovery (Bailey and Ollis 1986). The albumin protein background also complicates the downstream processing of protein products present in low concentrations. Aside from the safety issues, lot-to-lot variation can result in a lack of reproducibility of a process. The approval of a human gene therapy product may be expedited if it is produced under serum-free, protein-free conditions.

The use of serum-free medium often results in cells becoming adapted to suspension culture. The cells densities reached in suspension culture are often inferior to immobilised culture but there are still several advantages. Firstly, scale-up is relatively simple as with bacterial or yeast cultures. Secondly, cells can be grown in traditional fermenters. This does away with the need to validate new processes or reactors and may reduce capital costs. Thirdly, suspension cultures can be grown in a chemostat, which, due to the continual cell division, is anticipated to yield high virus titres (see 6.1).

6.5 Conclusions

On the basis of the work carried out, the recommended method for the large-scale production of retrovirus vectors for gene therapy applications is a perfusion culture using the fluidised bed bioreactor. Cytoline 1 microcarriers have proved to be a suitable substrate for cell growth. It is anticipated that virus titres can be further enhanced by reducing the culture temperature to 32°C and decreasing the serum concentration in the culture medium over the duration of the production run.

6.6 Recommendations for future work

Further work is required to evaluate the ability of TEFLYRD/83 cells to grow under serum-free conditions and the effect that this may have on virus production. Validation of suspension cultures is also required and a chemostat is suggested as an

alternative system for virus production. The ease and simplicity of suspension cultures has made them an attractive option in the large-scale production of biopharmaceutical products. Failure of these cells to grow in suspension may indicate the need for an alternative packaging cell line to be used in the production of OB83 retrovirus particles. HEK293 cells or PER.C6 cells are eligible candidates as they are proven cell lines for the production of recombinant retroviruses and can be grown in serum-free suspension cultures.

In this study, none of the immobilised culture systems examined were able to perform better than the roller bottle cultures. This may be due to the inability of virus particles to escape from the matrices and subsequently being denatured due to the prolonged time at culture temperature. The microenvironment within the particles may also be detrimental to virus particles as the pH would be lower than in the free supernatant, due to cell respiration. This again would have a negative affect on virus stability. Therefore, if cells could be grown in suspension, virus particles would be immediately released into the supernatant and would avoid the problems mentioned above. This also suggests that solid microcarriers may be more amenable to virus production, if cells are unable to grow in suspension, even though the cell densities may not be as high as those obtained with macroporous carriers.

If macroporous carriers are to be used in the future production of virus particles then more examination of the physical aspects pertaining to fluid flow and mass transport is required. An in depth analysis of packed bed reactors is required in order to understand the mechanisms that limit cell growth and inhibit the virus production rate, such as characterisation of fluid flow, the effect of channelling and the emergence of dead zones. Characterisation of cell growth, the particle microenvironment and the possibility of cell bridging is also required to fully understand these systems.

Chapter 7: Publications and Presentations

Papers:

Warnock J. N. and Al-Rubeai M. Production of Biologics in "Cell Immobilisation Biotechnology". Nedovic and Willeart (eds.). Kluwer Academic Publishers. (submitted).

Warnock J. N., Price T., Slade A., Al-Rubeai M. Alternative strategies for the production of retrovirus vectors. Biotechnology Progress. (submitted).

Warnock J. N. and Al-Rubeai M. Influence of serum concentration on cell growth and retrovirus production and decay kinetics. Cytotechnology. (submitted)

Presentations:

"Evaluation of Retrovirus Production Systems". ESACT-UK 12th Annual Meeting, Cambridge University, 3rd - 4th January 2002. Oral Presentation.

"Influence of serum on retrovirus production and decay kinetics". 15th Annual and International Meeting of the Japanese Association of Animal Cell Technology (JAACT) 2002, Fuchu, Tokyo, Japan. 11th-15th November 2002. Oral Presentation. Recipient of the JAACT Foreign Participants Award, and a Royal Academy of Engineering International Travel Grant.

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