CHAPTER 14

Three-dimensional cell-based assays in hollow fibre bioreactors

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Introduction

In many ways, the hollow fibre bioreactor may be considered the original three-dimensional (3D) cell culture system (Figure 14.1). Hollow fibre-based cell culture was first developed by Richard Knazek at the NIH in 1972. He was searching for a way to culture adrenal tumour cells under *in vivo*-like conditions to study hormone secretion in response to drug stimuli. His reports that response curves can be generated by assaying the amount of hormone secreted in response to biochemical stimuli were the original dynamic *in vitro* cell-based assay (Knazek *et al.*, 1972). Hollow fibre bioreactors offer a method by which cells can be cultured at tissue-like densities over long periods of time. Hollow fibres act as 'artificial capillaries' and act much as capillaries do in the human body. Hollow fibre bioreactors hit their peak of popularity in the late 1970s to 1980s when they were employed in the bio-manufacturing of monoclonal antibodies (Tharakan & Chau, 1986).

The controlled molecular weight cut-off (MWCO) of the fibres allowed inhibitory cytokines such as TGF-β to diffuse away from the hybridoma cells, while entrapping the secreted antibody to high concentration within the small volume of the extracapillary space. Poor flux in the particular fibres available at the time, coupled with a lack of available technology for the scale-up of hollow fibre bioreactors, limited their application and the method fell into disfavour, except for small-scale research applications.

In the past 10 years, advances such as new high-flux fibre materials, better system engineering and an improved understanding of hollow fibre cell culture methods have given new life to the use of hollow fibre bioreactors. It had been thought that the benefits of hollow fibre bioreactors were restricted to simply higher secreted protein concentrations, the ability to reduce serum levels or more easily adapt cultures to commercially available serum free media. Much

Technology Platforms for 3D Cell Culture: A User's Guide, First Edition. Edited by Stefan Przyborski.

Figure 14.1 Hollow fibre cartridge A hollow fibre cartridge represents a single-use, bio-safe closed system for culturing cells, bacteria and viruses. The standard sized cartridge has an extracapillary space volume of 20 mL and 3000 cm^2 of area.

new information has been gathered regarding hollow fibre bioreactors and it is now understood that they represent a fundamentally different and more *in vivo*-like way to culture cells. There is no question that the conditions under which cells are cultured have a profound effect on their behaviour, and cell culture conditions are fundamentally different in a hollow fibre bioreactor (Stanness *et al.*, 1997).

The hollow fibre bioreactor

The biomimetic hollow fibre (HF) bioreactor is a high-density continuous perfusion culture system. It presents many unique distinctions from the commonly employed non-porous plastic surfaces, for example flasks, microcarrier beads and discs or roller bottles. A HF bioreactor includes a cartridge containing thousands of semi-permeable hollow fibres in a parallel array within a tubular housing fitted with inlet and outlet ports (Figure 14.2). These fibre bundles are potted at each end so that any liquid entering the ends of the cartridge will necessarily flow through the interior of the fibres. Cells are generally seeded within the cartridge but outside the hollow fibres in what is referred to as the extracapillary space (ECS).

In this configuration, culture media is pumped inside the hollow fibres, allowing nutrients and waste products to diffuse both ways across the fibre walls. Having passed through the cartridge, the culture medium is oxygenated and recirculated to the cartridge. There are, however, a few other possible configurations and alterations of this basic implementation and flow circuit. Endothelial cells, the cells

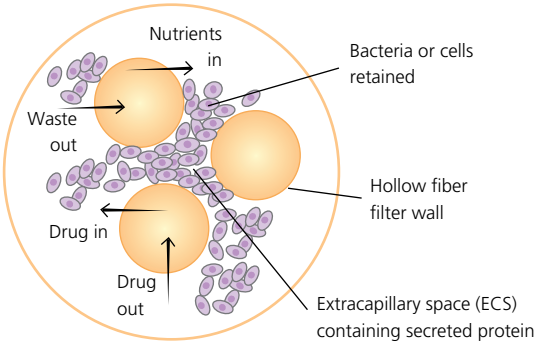

Figure 14.2 Cross-section of the hollow fibre cartridge. Medium is recirculated through the insides of the fibres and cells are typically grown on the outside of the fibres. The cells will attach to the porous support of the fibres and therefore do not require splitting or periodic subculturing.

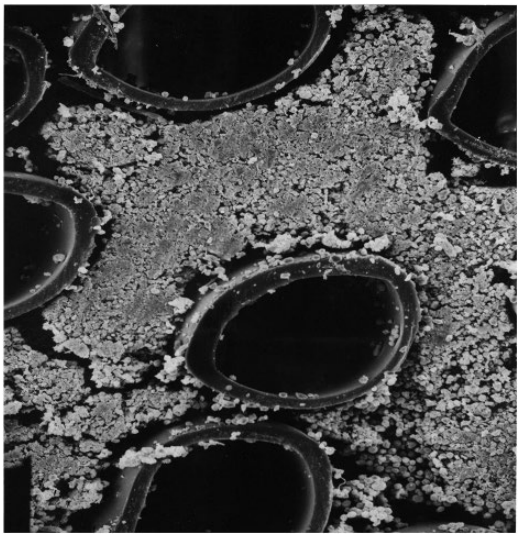

Figure 14.3 Lymphocytes cultured in a hollow fibre bioreactor. Lymphocytes are growing at very high densities, $1–2 \times 10^8$ cells per mL.

that line all blood vessels, can be seeded on the insides of the hollow fibres with the culture medium circulated over them. This flow provides hydrodynamic force (or shear stress) similar to what the cells would experience *in vivo*, and the cells respond to this shear.

Hollow fibre bioreactors offer a unique environment for a more biomimetic cell cultivation and disparate cell co-cultivation. They present a 3D environment similar to the conditions found in the body, and support the continuous control of parameters such as oxygenation levels, medium composition and drug concentration. HF bioreactors are an effective means for the generation of a number of products, from secreted proteins or viruses to cells or conditioned medium. There are three fundamental characteristics that differentiate hollow fibre cell culture from any other type.

- Cells are bound to a porous, perfused matrix much as they are *in vivo*.
- The MWCO of the support matrix can be controlled.
- A very high surface area to medium volume ratio (>150 cm^2 per mL) (Figure 14.3).

Cells bound to a porous support

The fact that the cells are bound to a porous support provides a number of distinct features. One is the support of a continuous system where there is no requirement to split the cells. Cultures in this system can maintain viability and production-relevant metabolism in a postconfluent manner for extended periods of time – months or longer. For example, one hybridoma cell line was reported to maintain productivity in a HF bioreactor-based culture for over one year. It has been reported that a contributing factor here is that more *in vivo*-like growth conditions afforded by HF bioreactors result in significantly reduced apoptosis (Hirschel *et al.*, 2011). Another advantage is that, due to the extremely low shear generated with the cartridge, the majority of cells that become necrotic will not release significant cytoplasmic proteins or DNA into the culture medium. This provides features such as an improved culture environment, more accurate culture parameter monitoring and a raw harvest that is cleaner and easier to handle for downstream assays and purification.

Controlled molecular weight cut-off

Through the selection of fibre porosity, desired products can be retained to significantly higher concentrations and the location/effects of cytokines can also be controlled. This is well illustrated in the case of hybridoma culture where the inhibitory cytokine TGF-β can be selectively removed from the culture while secreted antibody is concurrently retained (Dennler *et al.*, 2002). Secreted recombinant proteins can be selectively retained and concentrated and cytokines and other factors that facilitate cell-cell interactions can be concentrated as well. Small molecule drugs can easily exchange across the fibre and rapidly reach equilibrium.

High surface-to-volume ratio

The small diameter of the fibres (of the order of 200 microns) generates an extremely high surface area-to-cartridge volume ratio in the range of 100–200 cm^2/mL. In conjunction with the high gross filtration rate of polysulfone fibres, the exchange of primary and secondary metabolites is high enough to support cell densities of $1–2 \times 10^8$/mL, approaching *in vivo* tissue-like densities. A 20 mL cartridge will easily support the cell mass provided by a standard 2 L suspension culture or 20–40 roller bottles. High cell densities produce more protein per area of reactor footprint and facilitate adaptation to both lower serum concentrations and simplified serum-free culture, such as employing the FiberCell® Systems' CDM HD perfusion-optimised serum replacement. The use of such a defined and protein-free medium results in much cleaner product harvests and more simplified purification as well as a more defined culture environment compared to the presence of fetal bovine serum (FBS) (Figure 14.4).

Operation of a HF bioreactor, in its most simplified form, begins by seeding a prepared cartridge with either suspension or harvested adherent cells.

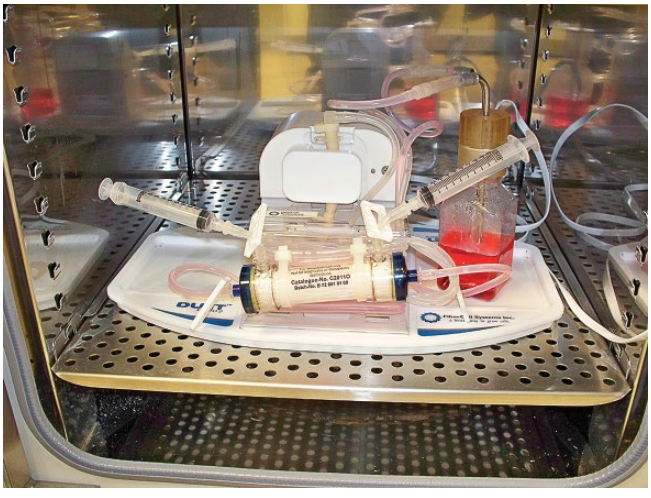

Figure 14.4 FiberCell Duet pump in a CO_2 incubator. Temperature and gas control is provided by the incubator and a thin cord is used to go through the incubator door without affecting gas composition.

The reactor cartridge is connected to an external reservoir and the medium recirculated from the reservoir through the cartridge. In one popular implementation, the recirculation is accomplished using a positive pressure displacement pump designed specifically for this purpose. Here, a piston compresses a short piece of pump tubing and one-way check valves on either side produce flow, in the same manner as the human heart. This frictionless pumping mechanism can generate 100 mL/minute of flow rate without wear on peristaltic pump tubing. Mass transfer of gases can be accomplished in a variety of ways, one being diffusive exchange through a loop of gas-permeable silicone tubing prior to the medium entering the bioreactor itself. The medium is constantly recirculated, providing a supply of oxygen and nutrients as well as removal of CO_2 and secondary metabolites. Medium recirculation rate and culture feeding can be linked to any number of culture parameters, and a number of control and automation options have been explored. A basic approach is to manually monitor the glucose and replace the medium as the concentration approaches 50% of the original.

Several cartridges sizes and fibre types are available, including those composed of polysulfone (PS) and polyvinylidene fluoride (PVDF). The MWCO in the PS fibres includes 5 KD or 20 KD and the pore size of PVDF is 0.1 micron. The PVDF fibre is of particular interest as various protein matrices, antibodies or growth factors can be readily bound to its surface via hydrophobic interaction. Cartridges come presterilised, assembled and ready to use and are intended for a single use.

During this renaissance of perfusion in general, and of the HF bioreactor in particular, several characteristics of hollow fibre cell culture have recently been identified.

- Reduction in apoptosis (Weeraphan *et al.*, 2012).
- Consistency of culture over long periods of time (G. Pavlakis, personal communication, 2013).
- More *in vivo*-like growth conditions resulting in improved cell function (Bennet *et al.*, 2007).
- Facilitation of the use of serum-free, protein-free and chemically defined media formulations (Whitford & Cadwell, 2011).

Hollow fibre bioreactors provide many particular (and some unique) culture characteristics due to a number of physical and ambient chemical conditions provided by the system, including:

- perfused medium flow and porous support permits long-term culture
- high cell density culture increases cell-cell contact
- selectable MWCO of fibres concentrates interactive cytokines
- selectable MWCO of fibres segregates cells, metabolites and products
- directional flow establishes gentle interstitial gradients within the cell mass
- hydrodynamic (shear) force on endothelial cells required for proper physiology
- long-term high-density culture on porous support facilitates development of cell-cell interactions over time.

Reduced apoptosis

It has long been recognised that reduced proteolysis and contamination by intracellular proteins and DNA are characteristics of the HF bioreactor. During extremely long culture periods of several months or longer, no degradation of secreted products has been reported, even when using serum-free and protein-free media. P.C. Liao's group has identified a potential mechanism for this (Wu *et al.*, 2009). Their research focused on the culture of primary cancer cells to identify potential secreted biomarkers. Cultures were initiated in FBS and then switched to a protein-free, chemically defined medium in order to reduce the amount of extraneous protein that might obscure the secretome of the cells. They found a significant reduction in apoptosis of up to 90%. The cells would die, but not lyse and go through the typical process of releasing proteolytic enzymes, intracellular proteins and DNA (Chang *et al.*, 2009). This, and the absence of significant shear forces inside the HF cartridge, results in dramatically reduced postsecretion alterations and cleaner harvests (Srisomsap *et al.*, 2010).

Culture consistency

In a HF bioreactor, the cells are bound to a porous support, not a non-porous plastic surface. Cell division rate and generation number are reduced, cultures do not require splitting, passage number is irrelevant, and cells grow in multiple layers in a 'postconfluent' fashion. Cultures can be maintained for many months and up to a year or longer (personal communication). Culture conditions remain

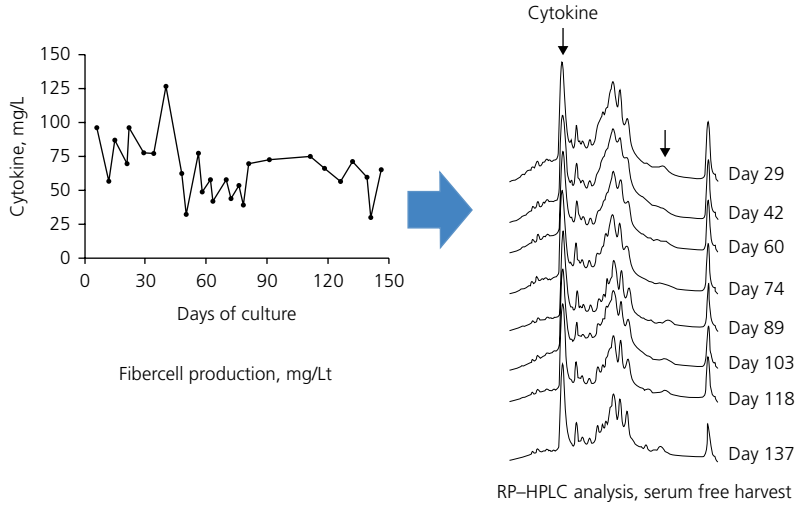

Figure 14.5 HEK 293 cells' cytokine product. HEK 293 cells expressing a heavily glycosylated protein in CDM HD protein-free medium. The HPLC demonstrates no changes in post-translational modifications over 137 days of continuous culture.

highly consistent during this time, as does cell physiology. In an example of this, provided by Dr George Pavlakis (NCI), 293T cells were transformed to produce a very complex protein. The cytokine product described was more than 40% carbohydrate and somewhat labile due to the fact that the two subunits are held together only with hydrophobic interactions. Attempts at producing this protein in standard flask or spinner cultures were unsuccessful. It was demonstrated that consistent production of dimerised product with complete and consistent post-translational modifications was maintained for over 140 days (Figure 14.5).

In vivo-like growth conditions

In vivo, most animal cells grow in 3D at very high density under tightly defined and highly controlled conditions. There is very little variation in oxygen tension, pH, glucose levels, etc. These parameters can have a wide range in flasks and other culture devices. It has been recently observed that 'The 2D cell culture systems used so far have several drawbacks: the morphology, proliferation, metabolism and expression profiles of cells grown in 2D systems are very different to cells in living tissues' (Tecan Group, 2012). It has been shown that in a HF bioreactor, consistency of culture conditions has a direct effect on cell physiology and product generation. One researcher found that transformed CHO cells expressing a hexamerised IgG responded favourably to HF bioreactor culture. The researcher first cultured the CHO cells in standard T150 flasks. Protein folding and post-translational modifications from this condition were incomplete, resulting in nearly 40% of the protein being expressed as an incompletely assembled monomeric subunit. When these same cells were removed from the

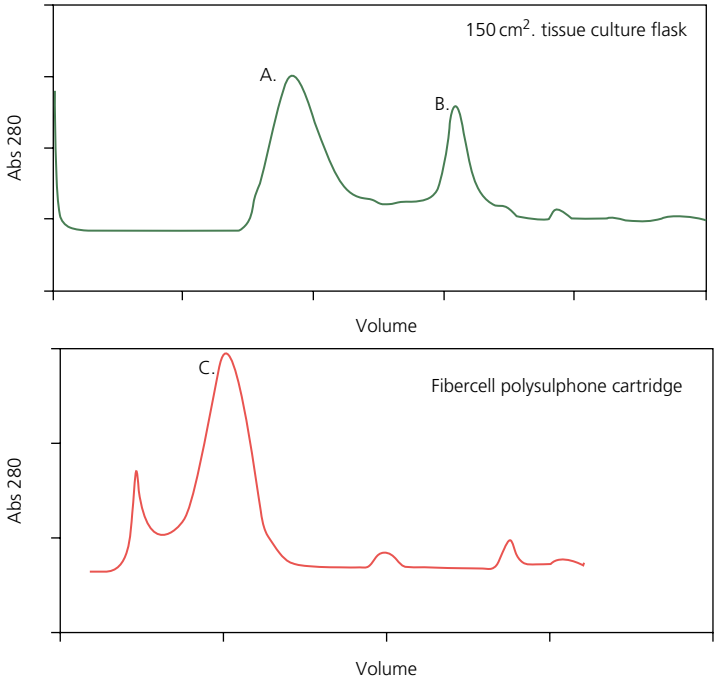

Figure 14.6 A CHO cell line expressing a hexamerised IgG protein construct. When these cells are cultured in flasks, 40% of the protein is expressed as a monomeric subunit. The exact same cells, when removed from the flask and seeded into the hollow fibre bioreactor, now express 95% of the protein as a properly folded hexamer.

flask and seeded into a HF bioreactor, within one week the expressed protein product was discovered to be 95% properly folded hexamer (J. Arthos, personal communication, 2013). The more biomemetic cell culture conditions had resulted in improved cell physiology and correct protein expression (Figure 14.6).

Single-use flow path systems

The culture-contact surfaces of many HF bioreactor instruments are composed of entirely single-use (SU) materials. These provide a number of benefits in the establishment of manufacturing and assay systems. They present reduced cross-contamination risks for many reasons, including that the materials have never seen living agents before and that they are sterilised by validated γ-irradiation systems. They provide lower initial investment costs as the SU housing apparatus is less expensive than full reuseable instrumentation. Lower facility and operating costs are supported by a reduced requirement for services such as autoclaves and process water as well as reduced operator requirements. Process efficiency and flexibility are provided by the ability to easily reroute the fluid flow path, and to order variant culture-contact materials or custom-designed assemblies from the manufacturer.

Protein-free, chemically defined culture medium

Besides providing many positive factors, animal serum presents a number of detrimental features including foaming, risk of contamination and variability in both production and cell-based assay performance. There are a number of non-culture-related limitations as well, including high and variable cost and the fact that high protein concentration can interfere with culture analysis. Cells *in vivo* are exposed to serum only during injury, and many cells then respond by particular activations. HF bioreactor culture characteristics, such as the very high cell density, allow for a reduction in serum concentration and facilitate adaptation to commercially available serum-free media. This has been taken one step further with the introduction of CDM HD, a commercially available serum replacement. CDM HD is a protein-free, chemically defined (CD), animal component-free serum replacement that is simplified and optimised for high-density cell culture.

Applications for cell-based assays

Many drug effects are dependent not only on concentration but also time. Some researchers believe cell-based assays and *in vitro* testing methods are a useful, time- and cost-effective tool for drug discovery. However, it is generally accepted that many of the available assays are not effective for examining the effects of both time and concentration, and do not closely mimic physiological kinetics. More specifically, such tests poorly report pharmacodynamic actions (what a drug does to the body) and pharmacokinetic actions (what the body does to a drug). Static cell-based assays in plates, flasks or other formats do not readily permit changes in drug concentration, as would be seen in humans from administration, uptake, distribution, distal metabolism and elimination effects. Animal models generally do not provide the same drug kinetics as would be found in humans, many infections cannot be supported with an animal model and often the bacterial load is not high enough to reveal the emergence of resistance. HF bioreactor cartridges have continuous medium circulation supporting dynamic control of drug concentration over time and resulting in the mimicking of naturally occurring gradients in tissue drug concentration. A high surface area-to-volume ratio permits extremely rapid exchange of metabolites and pharmacoactive molecules between the central reservoir and cells growing in the relatively small ECS of the cartridge. Furthermore, the volume of this central reservoir can be easily adjusted to permit rapid and reproducible changes in drug concentration (Figure 14.7).

Simulation of the kinetics of multiple drugs can also be accomplished so drug-drug interactions and combination therapies, as well as transport and efflux, can readily be modelled. The system is compact enough that multiple cartridges can be conveniently manipulated in a relatively small space, providing

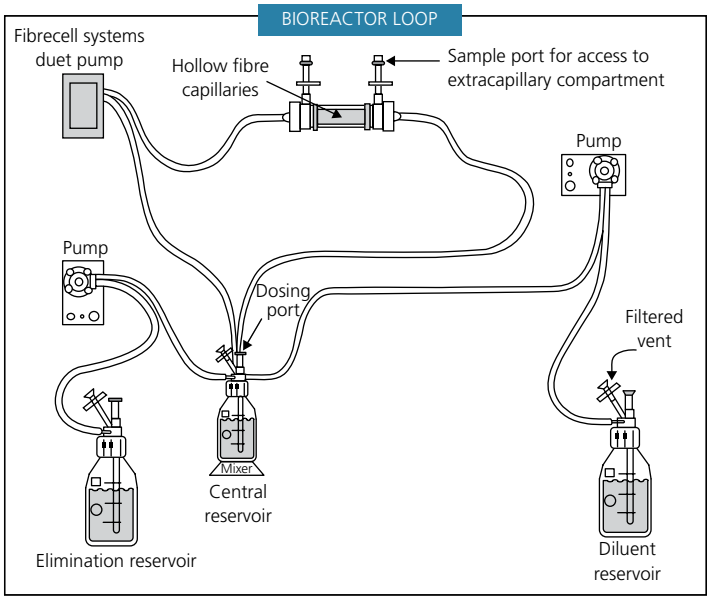

Figure 14.7 The set-up for controlling drug concentration to mimic human PK/PD. Medium or broth is constantly recirculating through the cartridge from the central reservoir. Drug can be introduced into the central reservoir at a defined rate representing the absorption curve. Diluent is then added and removed from the central reservoir at a controlled rate to model the elimination curve. Drug concentration in the extracapillary space of the cartridge equals the drug concentration in the central reservoir and the volume of the central reservoir remains constant.

multiplexed or parallel and higher throughput activities. Such systems can be configured for cell-based assays employing either a single-cell type or multicell in co-cultivation.

Examples of hollow fibre-based cell assays

Hollow fibre-based assays are inherently more complex and costly to design and set up than conventional cell-based assays. They require a higher degree of cell culture technical ability, more laboratory space and are more suited for higher value, more complex questions rather than smaller and truly high-throughput screening type assays. It can take up to a week before the culture is established and data can be generated. They are most effective when either using suspension cell types (permits the sampling of a small population of cells) or when measuring something the cells are secreting over time, such as a specific protein marker or virus particles.

However, these assays can generate data that is not available in any other manner, and can bridge an important gap between animal studies and phase I clinical trials. Large numbers of cells can be assayed over a pharmacologically

relevant period of time. Drug concentrations can be controlled in a dynamic fashion and both adsorption and elimination curves can be modelled. Multiple tests can be performed on the same cell population. 3D cultures of multiple cell types can model complex processes such as virus infections in tissues, haematopoiesis, cancer cell propagation, cancer cell metastasis and the blood–brain barrier. Furthermore, nuclear magnetic resonance (NMR) and other imaging methods have demonstrated potential as non-invasive ways to monitor the culture (Mancuso *et al.*, 1990).

Assays using only one cell type

The simplest type of 3D cell-based assays performed in HF bioreactors consist of only one cell type, seeded in the ECS. A popular implementation of this approach is in the study of antimicrobial compounds on bacteria. While outside the purview of this chapter, it is mentioned because it is the most common application of this format (Inloes *et al.*, 2004). The primary cell-based assays based upon this method are virus culture using a mammalian host cell and cancer cell-based assays.

There are many advantages for assaying drug effects on viral activity in a HF bioreactor. The rapid delivery of nutrients, removal of waste products and equilibration of drugs across the fibres facilitates the culture of cells at high densities. It is the only cell culture method that can support cells at physiological densities and provide for *in vivo*-like viral infection and pharmacokinetics at these densities. While both adherent and suspension cells can be cultured in a HF bioreactor, it is notable that adherent cells bound to a porous support do not require periodic splitting and can be maintained for extended periods of time. Suspension cells can also be supported for extended periods of time due to the same constant feeding and removal of metabolites.

The ability to add medium with or without drugs is particularly important for dose fractionation pharmacodynamic studies where compounds are added to the system over a short period of time and then removed by dilution with drug-free medium without disturbing the cells or their environment. Both absorption and elimination can be simulated and the small volume of the ECS contributes to the rapidity and economy with which this equilibrium takes place. Lastly, and perhaps most importantly, because of their large size both viruses and virus-infected cells are retained in the small volume of the ECS. These infectious agents cannot cross the fibres into the medium. The system is completely closed to the external environment and provides an added biosafety component protecting laboratory personnel from exposure.

Antiviral pharmacodynamics

Pharmacodynamics is the science that links drug exposure to response. A key element here involves identification of the best dosing and scheduling. This is derived from the principle that the drug concentration-time curve is a factor in a drug's effectiveness (Eagle *et al.*, 2004). For example, the duration that a drug

concentration remains above inhibitory levels may be closely linked to the antiviral effect. In this case, continuous or defined episodic intervals of drug exposure may lead to desired effects. On the other hand, peak concentrations of drug may be more important for antiviral activity. Here, brief doses at maximal concentrations may be most effective. It might also be demonstrated that the mode of dosing does not significantly affect antiviral activity. There are four essential pillars to the pharmacodynamic index (dose and schedule) of a particular entity for a particular virus:

1 The EC_{50}/EC_{95} values of the compound for the particular virus.
2 Binding (and therefore removal) of the compound to unreactive tissue and serum factors.
3 Identification of the compound's pharmacokinetics (and any variability of this within the population).
4 Identification of optimal exposure and administration schedule of the compound for the virus in question.

The high-density culture within HF reactors supports the efficient (biomimetic) cell-to-cell spread of virus in either matrixed or suspension cells. In either case, released virus and virus-infected cells accumulate in the ECS over time. Computer-controlled pumps administer drug and diluent into the central reservoir to model any schedule of drug exposure.

Antiviral effect can be determined by sampling the contents of the ECS through the sampling ports. This supports many divergent assays, beginning with virus-infected cell identification and counting by, for example, FACS analysis following treatment with a fluorochrome-labelled viral antigen-specific monoclonal antibody. The effect of drug on the yield of free infective virus particles can be determined by means such as plaque assay of harvested cell-free medium or by ELISA of viral antigen. The dosing regimen providing inhibition of viral replication and/or cell-to-cell spread of virus can be determined by sequential analysis of cell and ambient media viral titre and drug concentration from identified points later validated by, for example, LC/MS^n. Values for both drug exposure antiviral effects allow construction of an exposure–response relationship curve. Accuracy in this relationship is facilitated by the fact that eight or more evaluations can be performed simultaneously (McSharry *et al.*, 2011).

HIV

Nucleoside analogues

Nucleoside analogues are versions of the building blocks of DNA that almost act like normal nucleosides in DNA synthesis. But when these faulty building blocks are used, the new DNA cannot be built correctly. This in turn prevents the cell from producing new infective virus. Nucleotide analogue-based drugs also exist and are technically different from nucleoside analogues but act in much the same way. HF-based pharmacodynamic models have been employed in the examination of such HIV-1 antivirals for quite some time.

The nucleoside analogue 2′,3′-didehydro-3′deoxythymidine (d4T) was examined using HF infection models (HFIM) (Drusano *et al.*, 2002). Here, three HF units containing HIV-infected and uninfected CEM cells were set up. One unit was continuously infused with medium without drug and served as a no-drug control. A second unit received drug as a continuous infusion. A third unit received a bolus of d4T every 12 hours followed by a no-drug washout to model a one-hour pharmacokinetic half-life. In the absence of d4T there was a fourfold increase in p24 antigen over the nine days of the experiment. In the presence of d4T, delivered either as a continuous infusion or a twice-daily bolus, the amount of p24 antigen did not increase. In separate experiments, HF units infected with the same amount of virus and treated in the same way, but with d4T at half these doses, failed to completely inhibit virus replication. The results indicated the dosing required as either a bolus or continuous infusion to inhibit virus replication. The HFIM system predicted that the minimum effective dose of d4T to treat patients infected with HIV was approximately 0.5 mg/kg/day administered twice a day. This prediction was confirmed in a clinical study (Anderson *et al.*, 1992).

Protease inhibitors

The precursor Gag polyprotein is processed by a viral protease to many HIV structural proteins. A77003 impairs HIV-1 protease-mediated Gag processing and leads to an irreversible loss of the infectivity of the virus. A HF system was successfully used to determine the minimum concentration of the protease inhibitor A77003 that inhibits the replication of HIV in CEM cells (Preston *et al.*, 2003).

Vaccinia virus

Because routine smallpox vaccination among the public stopped after the disease was eradicated, the majority of the world's citizens are susceptible to infection with variola major virus. After the events of September and October 2001, however, the US government established goals for developing newer vaccines. Smallpox vaccine is made from vaccinia, a virus related to smallpox. Most smallpox vaccine contains live, replication-competent, vaccinia virus, not dead or attenuated virus like many other vaccines. For that reason, the vaccination site must be cared for to prevent the virus from spreading to other portions of the patient's body or other people. Also, the vaccine can cause undesirable side effects and complications.

The main challenge in developing a new, safer vaccine is that its effectiveness cannot be tested on humans, and other animals do not naturally contract smallpox. Monkeys or ferrets infected with other poxviruses are often used, but tests on animals that are artificially modelled with related disease often give false or misleading results. For improved pharmacodynamic studies on vaccinia virus, some researchers have employed HF-based models. One group developed such a model based upon used HeLa-S3 cells because they have been used to grow

Figure 14.8 Example of a set-up to run multiple cartridges and multiple assays simultaneously. The closed nature of the system permits different experiments to be run in the same incubator.

large amount of vaccinia virus and can be maintained as adherent or suspension cultures. Here, the effect of cidofovir on vaccinia virus replication in the HeLa-S3 cells was monitored by FACS analysis of virus-infected cells and by the production of infectious virus using a plaque assay (McSharry *et al.*, 2009a) (Figure 14.8).

Influenza virus

There are many distinct types of antiviral compounds effective against influenza virus. Licensed examples include the adamantanes and neuraminidase inhibitors. Amantadine has been used extensively for years, but recently almost all circulating influenza viruses have become resistant to it. Oseltamivir, a neuraminidase inhibitor, is now the most commonly used antiviral for uncomplicated influenza. The current recommendation for treatment with oseltamivir is to take two 75 mg tablets twice a day. Researchers wanted to determine if once-daily treatment was possible. They employed HF-based models to perform dose range

and dose fractionation experiments (McSharry *et al.*, 2009b). As is the case for poxviruses, there are no cell lines that continuously produce influenza virus infection. The standard cell line used for influenza viruses is the Madin–Darby canine kidney (MDCK), which grows well on glass and plastic surfaces and is permissive to various strains of influenza virus, but not with high efficiency.

To perform pharmacodynamic studies for oseltamivir and influenza viruses, this group used a derivative of MDCK that expresses higher levels of a particular cell surface glycoprotein than the original line. It was determined that the R292 strain of influenza virus grows well in these cells in the HF units. To perform dose-ranging studies in the HFIM system, six HF units containing influenza A virus-infected AX-4 cells were mixed with uninfected AX-4 cells and continuously infused with various concentrations of oseltamivir. The effect of the drug on virus replication was determined daily by plaque and haemagglutination assay. This provided an EC_{50} value in the HFIM system for oseltamivir with this isolate of the R292 strain of influenza virus. To determine the pharmacodynamically linked index, a dose fractionation study was designed to mimic a variety of half-lives of the drug in application. Here, one HF unit received an appropriate exposure delivered by continuous infusion; a second unit received the same exposure once a day followed by a no-drug washout; a third unit received the same exposure delivered twice a day followed by a no-drug washout; and a fourth unit received the same exposure delivered three times a day followed by a no-drug washout.

The data showed that in the absence of drug, the virus grew well in the HFIM system. The continuous dose and all three fractionated doses gave the same amount of inhibition of virus replication. This group concluded that the pharmacodynamically linked index for oseltamivir for the R292 strain of influenza A virus is the $AUC/EC_{50}/_{95}$ ratio. This means that the model indicates that at the appropriate dose, oseltamivir could be given once a day.

The demonstration that adherent cells can be used to grow virus in the HFIM system opens this system up to the pharmacodynamic analysis of antiviral compounds for a wide variety of viruses. As long as the virus-infected cells produce virus that is released into the ECS, then the effect of antiviral compounds can be measured, such as in H1N1 influenza (Brown *et al.*, 2011).

Anticancer agents

Another example of a 3D cell-based assay using only one cell type in the ECS of the HF cartridge is for the analysis and characterisation of anticancer agents. Anticancer agents also exhibit both time- and concentration-dependent efficacy and the goal of cell-based assays can be twofold. The first is to ascertain the most effective dosage profile for cytotoxicity of cancer cells while leaving normal cells in an acceptable state. The second is to provide an indication of the compound's robustness and application breadth by testing different types of cancer cell lines under identical conditions.

Kirstein reported on the use of the HF model for anticancer drug evaluations. In this case, gemcitabine was examined in the anchorage-dependent MDA-MB-231 breast cancer cell line (Kirstein *et al.*, 2006). Longer-term exposures are possible in continuous culture HF systems since the cells do not require splitting when a confluence is reached. More accurate results are obtained for a few reasons. One is that because the multilayer, 3D organisation of these continually perfused cells more closely reflects the *in vivo* structure of the tumour, they have an increased relevance for assays of anticancer agents. Another is that the cell division rates in static cultures are commonly artificially high, and this can render them more sensitive to some chemotherapeutic agents then their natural counterpart (Kirstein *et al.*, 2008).

Assays using more than one cell type

Hollow fibre and 3D co-cultivation for cell-based assays

Historically, the science of cell biology was intended to reduce complex systems to their most fundamental components in order to better understand them. However, in this age of systems biology, we are appreciating the limitations to such reductionist research, and better understanding the implications of organisms and tissues being made up of more than one type of cell. From the consequences of complex physical interactions to the signalling between multiple cell types, we are seeing the behaviour of cells in the context of an organisation or ensemble of heterotypes. HF bioreactor-based culture is one of the few *in vitro* techniques providing large numbers of cells in close enough proximity and sufficiently high density to observe a number of tissue-like behaviours (Figure 14.9). These behaviours include:

- signalling and direct interactions between different cell types
- co-ordinate (or synergistic) activity upon the ambient media
- co-ordinate (or synergistic) activity upon a compound or drug
- mixed cell type-specific effect (+/−) upon viral infections.

Hollow fibre cell culture permits the recapitulation of natural structures containing more than one cell type in a defined, controlled and more biomimetic environment. Endothelial cells are the only cells that can be easily cultured on the insides of the fibres under flow. There are two types of cell co-cultivation that can be performed in a HF bioreactor. They are one or more cell types:

- on both the inside and the outside of the fibres (Davis, 2007)
- on only one side of the fibres (typically on the ECS).

An example of the first type of cellular co-cultivation is the use of HF bioreactors to culture endothelial cells on the insides of the fibres while culturing a different cell type on the outside to provide cell signalling. Endothelial cells comprise the inner lining of all blood vessels, and the endothelium can actually be considered a discrete organ, rather than simply a cell type. Endothelial cells were first

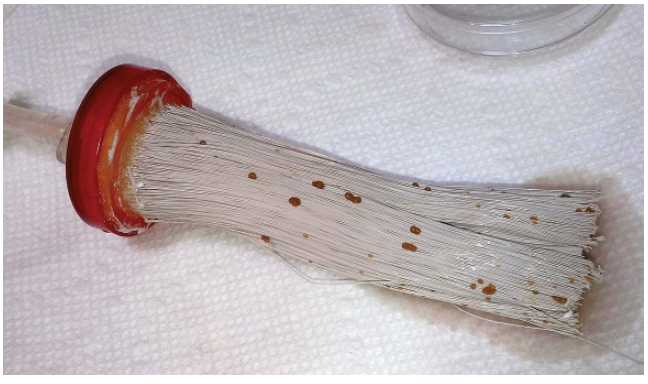

Figure 14.9 Co-cultivation of mixed cell population derived from collagenase digestion of a human placenta. This results in the formation of three-dimensional structures capable of generating suspension cells with stem-like phenotypes. Source: Courtesy of FiberCell Systems.

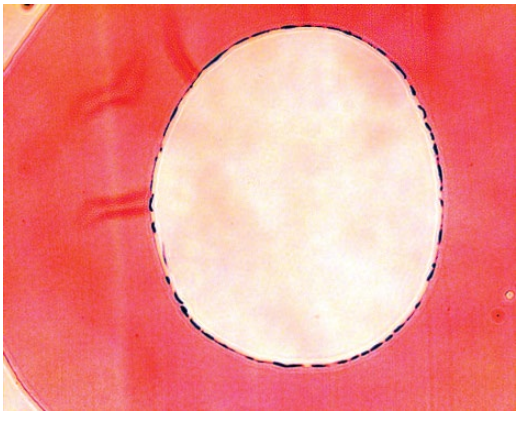

Figure 14.10 Cross-section of a PVDF fibre with an endothelial cell monolayer. This monolayer forms on the inside of the fibre under the influence of shear stress.

cultured on the insides of hollow fibres by Dr Barbara Ballerman (Ballerman & Ott, 1995). When cultured on the insides of a hollow fibre (the intracapillary space) and exposed to hydrodynamic shear stress from medium flow, endothelial cells behave differently (more *in vivo*-like) than they do in static, flask culture. Under shear, endothelial cells lay down flat as a monolayer, stop dividing, form tight junctions and express different genes than when cultured under static conditions in flasks (Figure 14.10). Differences in shear stress throughout the body may explain both the different functions and characteristics of endothelial cells in those areas (Redmond *et al.*, 1995).

The concept of 'organ recapitulation' in hollow fibres was first applied by Jorg Gerlach using liver tissue (Gridelli *et al.*, 2012). The system used was a complex HF bioreactor with two different fibre types and three separate bundles

of fibres. Liver tissue was simply digested with collagenase and this mixture of cells seeded into the HF bioreactor. Primary function was maintained for four weeks. It appeared that endothelial cells provided a necessary component of the cell mixture and were involved in the formation of 3D structures (Hoffman *et al.*, 2012).

Bone marrow model

Perhaps the most rigorous application of HF systems for single-compartment cell co-cultivation is in the area of stromal cell/suspension cell interactions. Contrary to popular belief, it is possible under specific conditions to harvest large numbers of healthy, intact cells from a HF bioreactor. In fact, the most powerful applications for HF systems involve longer-term cultures with periodic harvesting over time. There are a number of recent examples of this effectiveness presenting some very interesting results. In demonstration of the second type of co-culture, Dr Mayasari Lim at University Hospital, Hong Kong, published an article on the co-cultivation of a human bone marrow stromal cell line with a leukaemic T cell line (Usuludin *et al.*, 2012). The standard approach to this is generally accomplished in flask culture, but here she reported the establishment of a stromal line cultured to fairly high density within one week of culture initiation in the HF cartridge. Once the glucose uptake rate of standard media in the cartridge reached 1 g per day, the leukaemic T cell line was seeded into the cartridge. When co-cultured in flasks, the T cells would typically undergo a 10× expansion. When cultured in the HF cartridge, the T cells underwent a 4000-fold expansion, much more in line with what occurs *in vivo* (Figure 14.11).

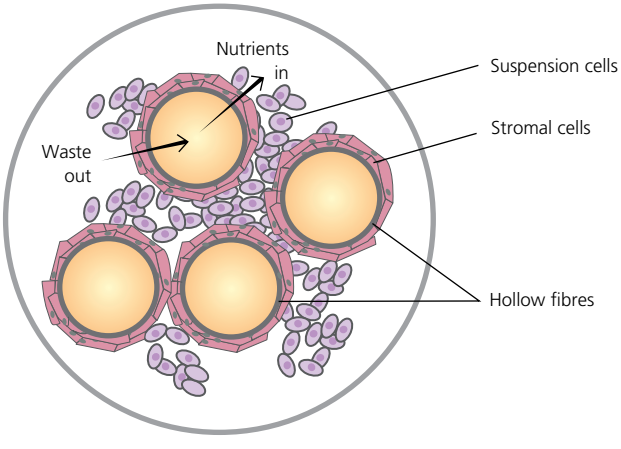

Figure 14.11 Schematic drawing of adherent stromal cells interacting with suspension cells. Since the cells are bound to a porous support, they do not require splitting and the culture can be maintained for several weeks to months.

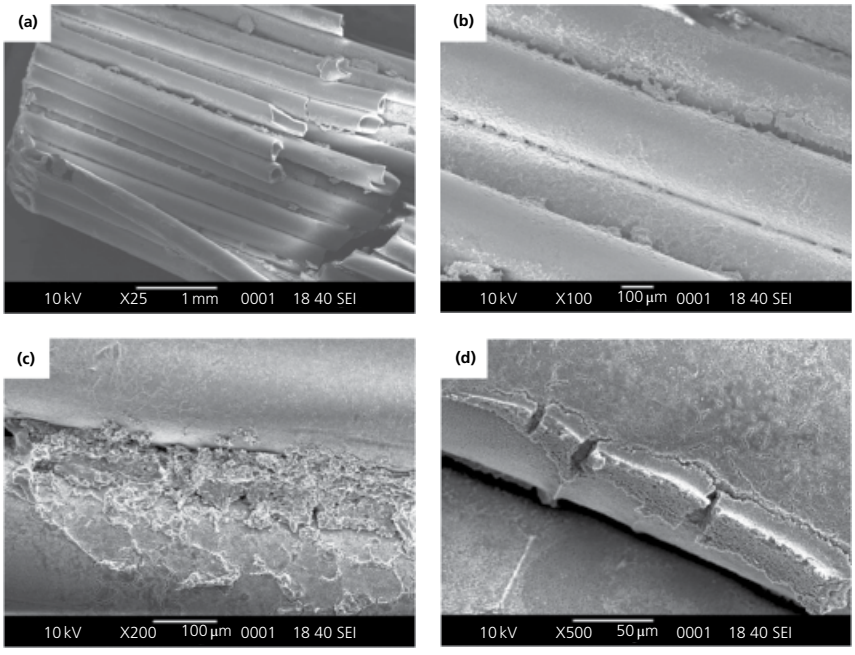

Figure 14.12 Scanning electron micrographs of bone marrow stroma cells bound to the surface of the 5 KD MWCO polysulfone fibres. These cells can interact with haematopoietic stem cells or leukaemic lymphocytes or other cell types. Images (a–d) are of increasing magnification showing the general structure of the polysulfone fibres with 3D cell growth in between.

The bone marrow microenvironment plays an integral role in the regulation of haematopoiesis. Residing stromal cells and the various extracellular matrixes in the bone marrow provide biological signals that control haematopoietic stem cell (HSC) function. In this study, a biomimetic co-culture platform using the HF bioreactor was developed for the *ex vivo* expansion of HSCs. The efficacy of such a platform was evaluated in comparison to standard cultures performed on tissue culture polystyrene (TCP). A human stromal cell line (HS-5) was employed as a co-cultured stromal support of lineage cell-depleted human cord blood cells. This was accomplished in serum-free medium supplemented with a cytokine cocktail (Xue *et al.*, 2014) (Figure 14.12).

Results showed that the performance of the HF bioreactor in supporting total cell and CD34+ progenitor cell expansion was comparable to that of cultures on TCP, while cells harvested from the HF bioreactor had a higher clonogenic ability. The performance of *ex vivo*-expanded cells from the HF bioreactor upon haematopoietic reconstitution in humanised mice was also comparable to that of the TCP control. Scanning electron microscopy revealed that stroma cell growth inside the HF bioreactor created a three-dimensional cell matrix. These findings demonstrate the feasibility of utilising an HF bioreactor for creating a complex matrix architecture, which may provide good *in vitro* mimicry of the bone marrow supporting large-scale expansion of HSCs.

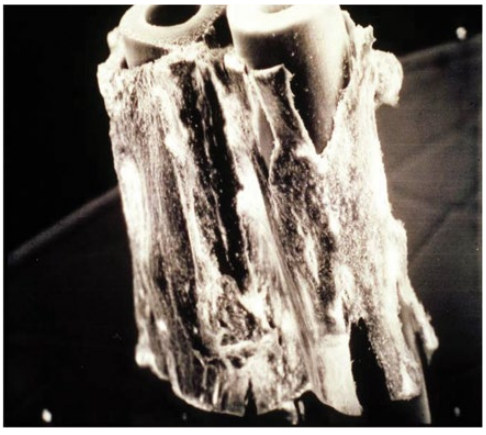

Figure 14.13 Scanning electron micrograph of bovine aortic endothelial cells. These cells were cultured on the insides of the fibres and vascular smooth muscle on the outside of the fibre. Note the 3D structure formed by the vascular smooth muscle cells. Source: Courtesy of Dr. Paul Cahill and Dr. Eileen Redmond.

Asymmetric co-culture using endothelial cells

Three-dimensional cell-based assays utilising co-culture of multiple cell types in HF bioreactors can recapitulate more complex structures than those with a single cell type. One type of cellular co-cultivation, as described above, is the use of HF bioreactors to culture endothelial cells on the insides of the fibres and a different cell type on the outside. A number of such examples have been reported, including some with significantly more complex asymmetric cell co-cultivation. For example, Paul Cahill cultured bovine aortic endothelial cells on the insides of the fibres and vascular smooth muscle cells on the outside of the fibres. As altering the flow rate changed the shear stress, G-protein formation and endothelin receptor expression were directly modulated, even though there was no physical contact between the two cell types (Redmond *et al.*, 1995) (Figure 14.13).

Blood–brain barrier model

Endothelial cells at the blood–brain barrier (BBB) demonstrate tight intercellular junctions, the absence of fenestrations and reduced pinocytotic vesicles. The *in vitro* study of the BBB has progressed rapidly of late as improved monitoring technologies and new or enhanced cell culture models have become available. Developments in *in vitro* models promise the design of new drugs for treatment of CNS disorders. These models play an important role in investigating the ability of such compounds to cross the BBB. There are many *in vitro* approaches to modelling BBB physical and biochemical behaviour, but most fail to represent its three-dimensional nature and do not support the associated exposure of endothelial cells to such complex influences as exist *in vivo* (Janke *et al.*, 2013).

To answer this challenge, Janigro developed a new, dynamic, *in vitro* BBB model (NDIV-BBB) designed to allow for extensive pharmacological, morphological and physiological studies (Stanness *et al.*, 1999). He first reported that brain microvascular endothelial cells developed robust growth and differentiation when cultured alone. In the presence of glial cells, they developed measurable properties allowing monitoring of the system and experimental

perturbations and physical manipulations. For example, it was noted that flow rates played an essential role in endothelial cell differentiation and concomitant decreased cell division. His new dynamic model of the BBB allows for longitudinal studies of the effects of flow and co-culture in a controlled and fully recyclable environment. It also permits visual inspection of the abluminal compartment and manipulation of individual capillaries. This model and testing services are available from FloCel (www.flocel.com).

In perhaps the ultimate embodiment of state-of-the-art HF-based cell assays, Dr Chris Pepper and co-workers have developed a model for primary investigations and assaying for cancer metastasis (Walsby *et al.*, 2014). It utilises endothelial cells on the inside of the fibre subjected to shear stress as well as their interaction with circulating leukaemic lymphocytes (Figures 14.14 and 14.15). There is

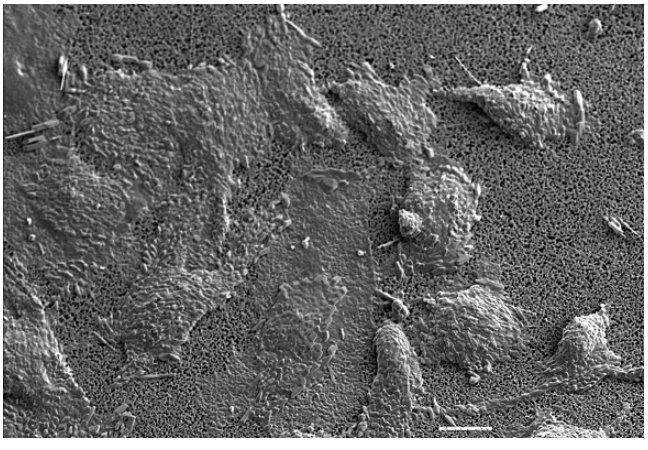

Figure 14.14 Endothelial cells attached to the inside of a PVDF hollow fibre with a short period of shear stress. Cells have begun to flatten in response to the shear.

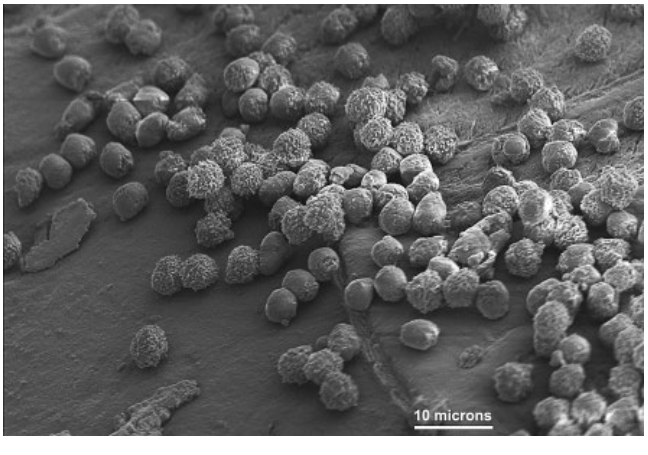

Figure 14.15 Circulating leukaemic lymphocytes on the outside of the fibres. This was taken after the transmigration of lymphocytes and their specific interaction with endothelial cells on the insides of the fibres.

growing evidence that lymphocyte trafficking contributes to the clinical course of chronic lymphocytic leukaemia (CLL) but to date only static *in vitro* cultures have been used to study these phenomena. To address this, a dynamic *in vitro* model was developed in which CLL cells experience shear forces equivalent to those in capillary beds and are made to flow through capillary-like hollow fibres lined with endothelial cells. CLL cells treated in this way increased their expression of CD62L, CXCR4, CD49d and CD5 directly as a result of the shear force. Furthermore, CLL cells migrated through the endothelium into the 'extravascular space' (EVS). Migrated CLL cells had significantly higher expression of CD49d, MMP-9, CD38, CD80 and CD69 compared with CLL cells that remained in the circulation. The degree of migration observed strongly correlated with CD49d expression, and treatment with the CD49d blocking antibody natalizumab resulted in significantly decreased migration. Taken together, these data provide evidence for a novel, dynamic and reproducible *in vitro* model of lymphocyte migration and cancer metastasis. This model might also prove useful in the study of stem cell homing and trafficking.

Conclusion

As the original 3D culture approach, HF systems have for decades supported the biomimetic culture of many cell types, at tissue-like densities, for extended periods of time. Novel material features supporting a renaissance in the technology include fibres composed of new materials, new surface derivatisations and porosities providing improved binding, flux and flow rates. Enhanced process control features include innovative culture monitoring approaches, automated feed and recirculation control as well as improved process understanding leading to new closed-loop control parameters. New and innovative application development has kept pace with the availability of these new technologies, expanding the scope of applicability of 3D HF cell-based assays. The HF bioreactor represents a more *in vivo*-like method by which to culture cells for assays. The new fields of metabolomics and systems biology are supporting the development of HF systems based upon multicell co-culture and defined serum-free media. The result is a robust and flexible technology with diverse applications. Such applications range from protein biological production providing ultra-clean harvest and simplified purification to improved *in vitro* viral infection models providing more accurate drug candidate pharmacokinetic/pharmacodynamic modelling.

The 3D HF-based cell assay system may not replace either animal models or human testing but does represent an important methodology for the testing of clinical compounds. It provides human kinetics and pathogen load levels before or in conjunction with human testing and bridges an important gap between animal and human testing.

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