3D CELL CULTURE
developments in technology to improve *in vitro* analyses

Improved *in vitro* models are required to aid the identification and assessment of candidate molecules for pharmaceutical development. Conventional cell culture models involve the growth of cells on two-dimensional (2D) substrates. Cells adapt to this synthetic 2D environment, become flattened and behave in an aberrant fashion. There is now significant demand for new three-dimensional (3D) cell culture models which allow cells to grow and adapt to their environment in a manner that more closely represents that experienced by their native counterparts. There are numerous advantages in enabling cells to acquire a natural 3D phenotype, including increased cell proliferation, differentiation and function. This article provides a brief overview of some of the technologies and approaches developed for 3D cell culture.

By Prof Stefan Przyborski

The cost developing a new pharmaceutical product has risen substantially in recent years, especially as the development of a new compound heads toward market and enters expensive animal testing and clinical trials. The losses incurred as a consequence of compound failure during late stage development can amount to multiple millions of dollars or even the collapse of a company, particularly in the biotech sector. There is now an urgent call for more appropriate assays that reduce this risk and enable investigators to make informed strategic decisions to be made earlier rather than later. There is also the need to mine new model systems and increase the strike rate at identifying new lead compounds. In general, *in vitro* studies are largely less expensive, faster and more flexible than regulated *in vivo* tests. However, rising cost-to-delivery ratios and poor predictive value of existing *in vitro* tests places great emphasis on the development of more realistic models, in particular the improvement of current cell culture assays.

Demand for 3D cell culture technology

Mammalian cell culture enables scientists to investigate cell function, model disease and screen compounds and develop new therapeutic approaches. Such technology is employed worldwide in academic institutions and in the healthcare, biotechnology and pharmaceutical industries. It is predicted that the use of these techniques will increase as researchers look for new ways of studying cells in the laboratory. Not only is there the drive to improve the quality of data generated from such assays, there are also external pressures such as improving efficiency and decreasing the cost of the R&D process. In addition, changes to policy and legislation that govern the use of animals in research and the need to reduce animal usage consequently impact on the development of alternative *in vitro* methods. As a result, the cell culture technology has huge market potential and is recognised as a billion dollar industry[1].

Conventional cell culture involves the growth of cells on flat 2D substrates which is synthetic and far

References

1 Cell and Tissue Culture Supplies: Marketing Report. Global Industry Analysts Inc. (2006).

2 Ben-Ze've, A (2005). Animal cell shape changes and gene expression. BioEssays, 13, 207-212.

3 Schmeichel, KL, Bissell, MJ (2003). Modeling tissue-specific signalling and organ function in three dimensions. J Cell Sci, 116, 2377-2388.

4 Bhadriraju, K, Chen, CS (2002). Engineering cellular microenvironments to improve cell-based drug testing. Drug Dis Today, 7, 612-20.

5 Sun, T, Jackson, S, Haycock, JW, MacNeil, S (2006). Culture of skin cells in 3D rather than 2D improves their ability to survive exposure to cytotoxic agents. J Biotechnol, 122, 372-381.

6 Zimmerman, H, Shirley, SG, Zimmerman, U (2007). Alginate-based encapsulation of cells: past, present, and future. Curr Diab Rep, 7, 314-320.

7 Dvir-Ginzberg, M, Elkayam, T, Cohen, S (2008). Induced differentiation and maturation of newborn liver cells into functional hepatic tissue in macroporous alginate scaffolds. FASEB J, 22, 1440-1449.

8 Blackshaw, SE, Arkison, S, Cameron, C, Davies, JA (1997). Promotion of regeneration and axon growth following injury in an invertebrate nervous system by the use of three-dimensional collagen gels. Proc Biol Sci, 264, 657-61.

9 Sakiyama, SE, Schense, JC, Hubbell, JA (1999). Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering. FASEB J, 13, 2214-24.

10 Yu, X, Dillon, GP, Bellamkonda, RB (1999). A laminin and nerve growth factor-laden three-dimensional scaffold for enhanced neurite extension. Tissue Eng, 5, 291-304.

Continued on page 71

removed from the environment which cells experience in living tissues. One of the key differences between these two growth conditions is the impact of the environment on the physical shape and geometry of the cell. *In vivo*, cells have evolved to acquire their natural 3D structure, that is optimal for their normal growth and function. Furthermore, cells are supported by a complex 3D extracellular matrix (ECM) which facilitates cell-cell communication via direct contact and through the secretion of cytokines and trophic factors. Many of these factors are changed when cells are extracted from living tissues and explanted into 2D cell culture where they are generally confined in 2D monolayers without many of the physical and chemical cues which underlie their identity and function *in vivo*. The scope for cells to adopt natural morphologies or to communicate efficiently with their neighbours is significantly reduced in 2D culture. It has been known for many years that cell shape and contacts influence the cytoskeleton which in turn can regulate gene and protein expression and hence cell function[2]. As a consequence, it is widely recognised that this 2D confinement is far removed from the aforementioned 3D complexities in living tissues and that this impacts on the validity of the data generated from 2D cell culture models.

The scientific literature describes many instances in a broad range of applications where 3D cell growth is different and is advantageous over conventional 2D culture. For example, it has been shown that the growth and function of cells as multi-cellular 3D structures is significantly different to their growth as conventional 2D monolayer cultures[3]. Furthermore, engineering the cell culture micro-environment to create growth conditions that more accurately mimic the *in vivo* behaviour of cells is an essential step for improving predictive accuracy during drug discovery[4]. The design of 3D culture systems for use in pharmaceutical development is an important part of this process. Data show that refinement of the *in vitro* environment significantly influences the way in which cells respond to small molecules[5].

A now overwhelming amount of evidence suggests that enabling cells to grow in a 3D spatial environment will overcome some of the restrictions associated with 2D cell culture. As a consequence, a significant amount of effort is now focused on engineering materials to create such a 3D space for cell growth, which will begin to bridge the gap between conventional 2D cell culture and living tissue environments. This article introduces some currently available technologies that enable 3D cell culture.

Technologies that enable 3D cell culture

A search of the scientific literature will reveal that there are many different approaches that enable the growth of cells in 3D. Traditionally, 3D cell growth has been an aspiration of tissue engineers, particularly for the generation of tissues to be used in transplantation. Comparatively less attention has been devoted to the development of technology for 3D culture for exclusive application in the laboratory. Furthermore, very few examples have been developed commercially into products that are readily available 3D cell culture technologies designed to improve the accuracy of *in vitro* analyses in a routine and cost-effective manner. In response to demand and interest in fabricating materials for 3D cell culture growth, there are now a number of basic approaches that can be categorised. These include naturally occurring materials as well as products fabricated from naturally-derived and synthetic polymers.

Natural scaffold substrates

Alginate is a seaweed-derived material that has been used as a natural substrate to support the growth of cells in a number of ways including cell encapsulation[6]. Alginate materials have also been developed into macroporous scaffolds which have been employed to support the development of 3D aggregates or 'spheroids' of hepatocytes[7]. Related technology has been developed commercially in the form of Algimatrix™ (Invitrogen). This is promoted as an animal-free 3D substrate for the development of high-fidelity cell culture models that enhance the predictive of drug responses in certain disease states. Algimatrix™ enables 3D cell growth, however, the growth of cells as individual spherical masses may not be suitable for all applications. Furthermore, the distribution of cells throughout the material is not entirely uniform and there are issues about the thickness of the scaffold in relation to mass transfer of oxygen, nutrients and waste products, particularly in the absence of media perfusion. While cell growth on alginate-based materials may have certain advantages, it is not clear how cultured mammalian cells that have been studied in contact with polystyrene for many years will respond to an alginate substrate.

Hydrogels

The use of hydrogels has been established for many years and are a common form of material used to support 3D cell growth *in vitro* for a broad range of tissues including bone, cartilage and nervous tissues[8-12]. As would be expected

from the name, hydrogels possess high water content but also contain cross-linked natural base materials such as agarose, collagen, fibrin or hyaluronic acid. The consistency of such gels can be modified to support preferential cell growth and function. Hydrogels surround cells in an artificial extracellular matrix environment and can include the incorporation of biological active molecules such as laminin[12], and changing the physical parameters of the scaffold such as charge[13]. Several hydrogel-based products are available commercially such as Matrigel™ (BD Biosciences), a popular animal derived product, and Extracell™ (Glycosan Biosystems), a chemically defined hyaluronan-based substrate. Injectable hydrogels have proven to be successful for tissue repair[8]. Hydrogels have been used to support 3D cell culture including the classic tubule and duct cell assay[14]. Such gels are often used as a film within which a degree of 3D cell culture can occur but their routine use may be restricted by various practical issues including expense, shelf-life, gel preparation and inconsistency.

Biodegradable materials

One of the early most successful approaches to enable 3D cell growth involved the use of biodegradable polymers such as poly(glycolic acid), poly(lactic acid) and their co-polymers (poly(lactic-co-glycolic acid)[15]. These types of material have proven useful during transplantation, for example, to encourage tissue regeneration and repair of articular cartilage[16]. Degradation of such materials occurs over time enabling integration of co-transplanted cells with surrounding host tissues. However, degradation of materials and release of products can result in changes in the chemical balance of the local micro-environment which can impact on cell growth. For routine 3D cell culture, biodegradability is not necessarily a useful feature and there are shelf life issues where improper storage of a biodegradable product can render it useless. Furthermore, investigators generally try to reduce the number of variables in an experiment to focus on a key factor, however, this is more difficult in an *in vitro* study where the scaffold is degrading and potentially influencing how the cells are functioning.

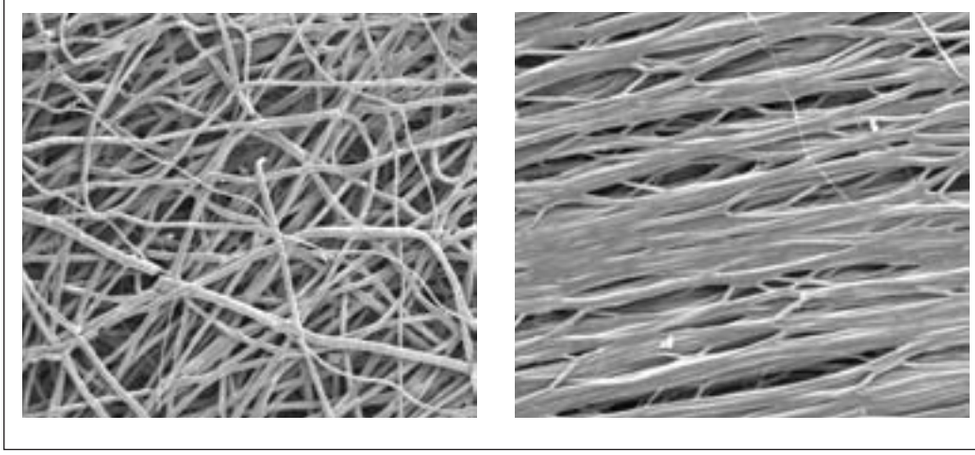

Figure 1: Structure of polystyrene electrospun fibre mats as demonstrated using scanning electron microscopy. Fibres can be generated in a random (left) or aligned (right) orientation

Inert non-degradable scaffolds

Inert non-degradable scaffolds consist of pores or voids which are joined by interconnecting holes into which cells can grow. They are most often developed from synthetic polymers and their simplicity overcomes several of the limitations that other 3D substrates may experience. There are numerous methods of fabricating porous materials including emulsion templating[17,18], leachable particles[19] and gas foaming technology[20]. Gas-in-liquid foam templating has been used as a method to create porous scaffolds for cell culture applications[21]. In this process gas bubbles can coalesce leading to a broad range of scaffold porosities in which it is difficult to control the consistency of the material and consequently the reproducible growth of cells within the scaffold.

Electrospinning is technology that has been developed heavily in the textile industry. The principles behind electrospinning have been applied to produce mats of electrospun synthetic fibres designed to support 3D cell growth[22]. While the consistency and porosity of electrospun materials is a challenge to control, they have the potential advantage of orientating cell growth. Fibre mats can be spun either in a random fashion or in a manner where fibres are aligned in parallel presenting directional passage for guided cell growth (**Figure 1**). Electrospun materials can either be used to provide a physical 3D space in which cells grow (although the uniformity of such materials is difficult to control) or they can be used to introduce topographical features upon which cells adhere. For example, Ultra-Web™ (Corning) was developed as a commercial polyamide elecrospun nanofibre mat for cell culture. Cells grow as monolayers on the roughen topography created by the nanoscale Ultra-Web™ fibre mat rather than within the physical lattice of the material.

Fabrication technologies such as those described have been applied to the manufacture of porous polystyrene-based scaffolds. Polystyrene is chemically inert, stable and is consistent and directly comparable to conventional 2D tissue culture plastic ware. These features make it an attractive medium as a scaffold to support 3D cell culture. The vast majority of *in vitro* cell culture experiments and resulting data have been conducted on polystyrene surfaces in one form or another. The transition from 2D to 3D cell culture is a major step change. However, the development of polystyrene-based scaffolds will ease the impact this has because the substrate remains the same and it is only the geometry of the polystyrene substrate which has changed from 2D to 3D. Polystyrene scaffolds are also beneficial given that they are designed as a consumable product with a long shelf life and they are generally simple and inexpensive to mass produce. These attributes make polystyrene-based scaffolds well suited for routine 3D cell culture.

Emulsion templating has been developed as a method to manufacture porous polystyrene that can subsequently be tailored to support 3D cell culture[17,18]. Alvetex™ (Reinnervate) is a new product that utilises this technology resulting in a polystyrene-based scaffold that has a uniform structure (**Figure 2**). The scaffold has been engineered into a thin 200 micron membrane to address the issue of mass transfer, enabling cells to enter the material

Continued from page 68

11 Fisher, JP, Jo, S, Mikos, AG, Reddi, AH (2004). Thermoreversible hydrogel scaffolds for articular cartilage engineering. J Biomed Mater Res A, 71, 268-74.
12 Park, Y, Sugimoto, M, Watrin, A, Chiquet, M, Hunziker, EB (2005). BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel. Osteoarthritis Cartilage, 13, 527-36.
13 Dillon, GP, Yu, X, Sridharan, A, Ranieri, JP, Bellamkonda, RV (1998). The influence of physical structure and charge on neurite extension in a 3D hydrogel scaffold. J Biomater Sci Polym Ed, 9, 1049-69.
14 Arias, AE, Bendayan, M (1993). Differentiation of pancreatic acinar cells into duct-like cells in vitro. Lab Invest, 69, 518-530.
15 Mikos, AG, Sarakinos, G, Leite, SM, Vacanti, JP, Langer, R (1993). Laminated three-dimensional biodegradable foams for use in tissue engineering. Biomaterials, 14, 323-30.
16 Temenoff, JS, Mikos, AG (2000). Injectable biodegradable materials for orthopedic tissue engineering. Biomaterials, 21, 2405-2412.
17 Bokhari, M, Carnachan, R, Przyborski, SA, Cameron, NR (2007). Effect of synthesis parameters on emulsion-templated porous polymer formation and evaluation for 3D cell culture scaffolds. J Mat Chem, 17, 4088-4094.
18 Carnachan, RJ, Bokhari, M, Przyborski, SA, Cameron, NR (2006). Tailoring the morphology of emulsion-templated porous polymers. Soft Matter, 2, 608-616.
19 Aydin, HM, El Haj, AJ, Piskin, E, Yang, Y (2009). Improving pore interconnectivity in polymeric scaffolds for tissue engineering. J Tissue Eng Regen Med, 3, 470-476.

Continued on page 72

Continued from page 71

20 Salerno, A, Oliviero, M, Di Maio, E, Iannace, S, Netti, PA (2009). Design of porous polymeric scaffolds by gas foaming of heterogeneous blends. J Mat Sci Mater Med, 20, 2043-2051.
21 Barbetta, A, Gumiero, A, Pecci, R, Bedini, R, Dentini, M (2009). Gas-in-liquid foam templating as a method for the production of highly porous scaffolds. Biomacromol. 10, 3188-3192.
22 Sun, T, Norton, D, McKean, RJ, Haycock, JW, Ryan, AJ, MacNeil, S (2007). Development of a 3D cell culture system for investigating cell interactions with electrospun fibers. Biotechnol Bioeng, 97, 1318-1328.
23 Dash, A, Inman, W, Hoffmaster, K, Sevidal, S, Kelly, J, Obach, RS, Griffith, LG, Tannenbaum, SR (2009). Liver tissue engineering in the evaluation of drug safety. Expert Opin Drug Metab Toxicol. 5, 1159-1174.
24 Bokhari, M, Carnachan, R, Cameron, NR, Przyborski, SA (2007). Novel cell culture device enabling three-dimensional cell growth and improved cell function. Biochem Biophys Res Comm, 354, 1095-1100.
25 Bokhari, Carnachan, Cameron, NR, Przyborski, SA (2007). Culture of HepG2 liver cells on three dimensional polystyrene scaffolds enhances cell structure and function during toxicological challenge. J Anat, 211, 567-76.

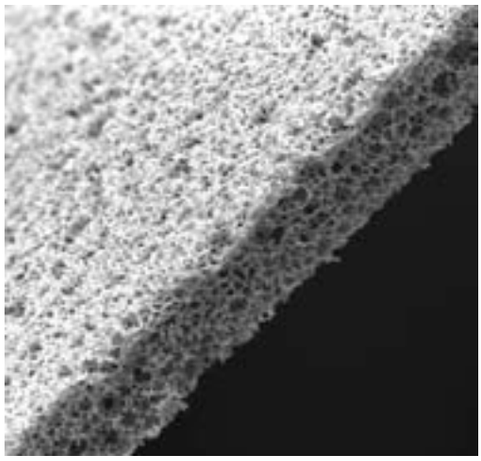

Figure 2: Emulsion templating can be used to generate highly porous materials such as this Alvetex™ polystyrene scaffold. The scaffold has been engineered into a 200 micron thick membrane shown in transverse section as imaged by scanning electron microscopy

and allowing for sufficient mass exchange of gases, nutrients and waste products during static culture. Alvetex™ membranes are designed to be incorporated into existing cell culture products, such as welled plates or well inserts. Furthermore, like conventional 2D plastic ware, polystyrene-based scaffolds are compatible with standard cell culture plasma treatment, gamma sterilisation methods and if required can be coated using standard cell culture reagents such as collagen, fibronectin, etc.

Conclusions

For 3D cell culture to become routine, the development of any new technology must consider issues such as cost, ease of use, application and reproducibility, especially when the application is for drug discovery. A technology that is expensive, difficult to use or is inconsistent in some manner, will not satisfy these demands and will fail to be accepted by the scientific community. Importantly, any such technology requires vigorous exemplification and validation as evidence of its ability to support true 3D cell culture over a range of alternative cell types. For example, cultured liver cells are a valuable tool for the *in vitro* study of drug metabolism and toxicity[23]. Liver-derived cell lines represent a convenient model for liver toxicology studies, although commonly available lines often display poor metabolic responses when challenged with a toxicant. Growing hepatocytes in 3D using scaffold technology, such as the application of Alvetex™ (**Figure 3**), can significantly enhance such responses[24,25]. Culture systems that show improved metabolic activity and/or more realistic resistance/sensitivity in response to specific drugs would be of significant value to the pharmaceutical industry, enabling more accurate toxicological assays and increasing the predictive accuracy during compound screening.

In general the growth of cells on conventional 2D plastic substrates has not changed significantly for many years. New innovative ways of culturing cells are becoming available that will improve current practice, cell growth and performance. The evidence demonstrating the advantages of 3D cell growth is compelling, as is the need for technology that enables routine 3D cell culture. The investment of time towards developing and validating such *in vitro* models is likely to significantly impact on the success and overall efficiency of pharmaceutical development in the future. DDW

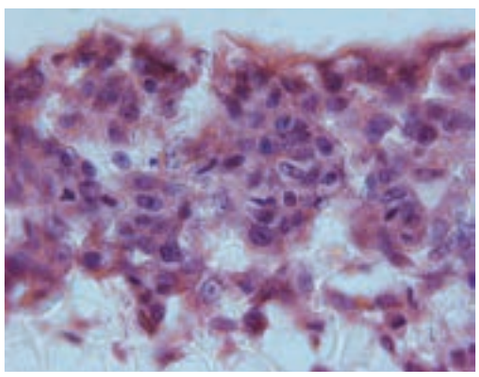

Figure 3: Micrograph showing the structure of liver hepatocytes (HepG2) grown in 3D culture on a porous polystyrene scaffold. Sample prepared by histological methods and H&E counterstaining

Professor Stefan Przyborski *is the Founder and Chief Scientific Officer of Reinnervate Limited. He also holds an academic position at Durham University as Chair in Cell Technology. He has more than 18 years' experience in cell biology, he manages a busy research group developing enabling technology to improve the growth and function of cells* in vitro. *Stefan publishes his work widely and frequently presents his research at scientific meetings.*