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The use of bioreactors as *in vitro* models in pharmaceutical research

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Keywords: *In vitro* model, bioartificial systems

Word Teaser

The development of dynamic 3D bioreactor based systems as *in vitro* models for use in DMPK studies.

Author Biographies

Maaria Ginai graduated with a first Class Bachelors degree with honours in Biomedical Science from the University of Kent (UK) in 2010. She is currently studying a BBSRC CASE-funded PhD at the Centre for Biological Engineering at Loughborough University in the area of bioartificial device progression to *in vitro* models for use in the pharmaceutical industry. This project is supported by AstraZeneca.

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Karen Coopman has a first Class Bachelors degree in Pharmacology from the University of Bristol (UK) and a PhD from the Department of Pharmacy and Pharmacology at the University of Bath (UK). She was appointed to a lectureship at Loughborough University, where she is the Operations Manager of the EPSRC-funded Doctoral Training Centre in Regenerative Medicine. She co-leads the Cell Technologies research group within University's Centre for Biological Engineering and is currently a member of the EPSRC's Early Career Forum in Manufacturing Research. The overarching themes of Karen's research are the manufacture of cellular therapies and the use of cells in the drug discovery process.

Abstract

Bringing a new drug to market is costly in terms of capital and time investments, and any development issues encountered in late stage clinical trials may often be due to *in vitro - in vivo* extrapolations (IVIVE) not accurately reflecting clinical outcome. In the discipline of drug metabolism and pharmacokinetics (DMPK), current *in vitro* cellular methods do not provide the 3D structure and function of organs found *in vivo,* so new dynamic methods need to be established to aid improvement of IVIVE. This review will highlight the importance of model progression into dynamic systems for use within drug development, focussing on devices developed currently in the areas of the liver and blood-brain barrier, and the potential to develop models for other organ systems such as the kidney.

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Currently, the pharmaceutical industry balances the stringent testing of drugs and products against soaring costs and return on investments risks. Bringing a new drug to market will, presently, on average cost $1.3 billion over 12 years [1] [2] with pre-clinical *in vitro* testing consuming around half of the total development time [2]. Moreover, many new chemical and biological entities (NCEs/NBEs) which fail late stage human testing give evidence that pharmacological and toxicity data from *in vitro* cell based assays are not always predictive of the clinical situation [3]. Due to these financial and time commitments, there is great emphasis within the industry to develop newer and more reliable *in vitro* and pre-clinical methods to accompany or replace the existing methods of NCE/NBE investigations.

In vitro models, particularly cell based versions are utilised in various areas of drug discovery, such as target identification and validation using disease models, compound screening and basic cytotoxicity using static cultures, through to ADMET (absorption, distribution, metabolism, excretion and toxicology) studies on lead compounds. While high-throughput cell based assays have revolutionised the efficiency and speed at which compounds can be screened, ADMET models ideally should be an accurate representation of the physiological or pathophysiological state, which may be lacking in 2D static cultures. Tissue engineering advances show promise in the development of models with organised structure and cell types similar to *in vivo*. However the dynamic aspect of *in vitro* models is yet to fully be addressed, although it is starting to be investigated with the use of three dimensional bioreactors seeded with cells.

Within the pharmaceutical industry the absorption, distribution, metabolism and excretion properties of NCEs are typically investigated as part of drug metabolism and pharmacokinetic (DMPK) science. These DMPK properties are optimized within drug discovery so that an NCE has the right clinical profile, e.g. ideally a high oral bioavailability, an elimination half-life that enables a once daily dosing regimen, sufficient exposure at the target tissue and an absence of drug-drug interaction (DDI) potential [4]. *In vitro* 2D cell

based models are routinely used within pharmaceutical research to investigate the DMPK properties of absorption (Caco-2, derived from human colon carcinoma cells), distribution (MDCKII-MDR1, derived from canine kidney cells) and metabolism/excretion (hepatocytes) for NCEs. More recently some three dimensional models have started to be investigated to assess their utility in studying aspects of ADME, namely the bioartificial liver (distribution/metabolism/elimination), the blood brain barrier model (distribution) and the bioartificial kidney (excretion). This review will therefore focus on the potential of bioartificial devices that have been developed for therapeutic use to progress to pharmaceutically relevant *in vitro* models for use in DMPK studies.

Limitations of current 2D systems

In vitro cell based models offer several advantages over the majority of cell free methods as they do not require purification of the target protein, they immediately select against compounds that are generally cytotoxic or cannot permeate cell membranes, and enable measurement of functional transport activity that is more representative of the physiological state [5]. Using primary human cells in these assays is the ideal gold standard [6]; however freshly isolated cells, cell cultures or tissue fragments cannot be used to study effects that occur over a longer time period due to the loss of functional integrity from drug transporter loss and enzyme depletion after a few hours [7][8]. In addition, primary human cells are a scarce and precious resource. For these reasons immortalised cell lines, such as (transfected) MDCKII-MDR1 or Caco-2 are often utilised in favour of primary cells. Such cells are relatively inexpensive and easy to propagate, especially in high throughput systems, however due to the genetic transformation process aspects of the original cell that are important to ADMET studies may be lost such as transporter expression and function, and cell adhesion molecules [8].

Although relatively simple and effective when using the appropriate cell type, '2D' *in vitro* cell based assays encounter the traditional problems of static culture; a constantly changing environment due to nutrient depletion and metabolic waste accumulation. Strategies to improve such systems have been introduced based on mimicry of the natural physiological environment of the organ in question. The reestablishment of heterotypic cell-cell and cell-extracellular matrix interactions through co-cultures and sandwich cultures of cells respectively have proven useful in the progression of *in vitro* model development for a number of tissue types including the liver [9] [10], brain [11] and lung [12]. These models show improved functionality and growth compared to their single culture counterparts, whilst keeping their ease of propagation and use compared to primary cell cultures, which is imperative when developing long term *in vitro* models for pharmaceutical applications.

Perfusion systems of isolated organs or tissue fragments offer the closest *ex vivo* model of the natural physiological state, alleviating the problems encountered with static

culture, maintaining 3D architecture and prolonging the functionality, heterogeneity, structural complexity and cell-cell interactions [13]. However, the shortage of organs available and the costs and difficulties of maintaining primary cells *ex vivo* make perfusion cultures unsuitable as a routine *in vitro* cell based system.

Moving towards 3D systems

The development of 3D constructs for pharmaceutical research now encompasses a variety of systems and devices. 3D cell culture with cell types native to a specific organ that are grown on natural scaffolds such as collagen, decellularised scaffolds or synthetic biodegradable scaffolds, present a bridge between 2D culture and the *in vivo* cellular environment. 3D devices for pharmaceutical research, with structural complexity and native functionality are now becoming available, however these cultures are difficult to maintain *in vitro* and still lack the vascular component of tissues. Extracellular matrix (ECM) coatings on scaffolds such as polymer scaffolds which release growth factors have been investigated to aid cell attachment, growth and partially represent the native environments which can have profound effects on the morphology, behaviour and functions of cells needed in drug discovery processes, but further work is needed to progress these cultures for use as models [14].

Bioartificial devices combine flow through systems, usually a form of simple plug flow bioreactor, with cells, thereby mimicking both the dynamic and cell specific aspects of native tissues [13]. They are being incorporated in the pharmaceutical industry due to the potential payoff of getting more drugs to market [15]. As will be discussed further in the following sections, these devices are being developed from clinically used systems, and whilst they offer cellular and environmental similarities, most systems currently lack the complex architecture and microenvironments seen in 3D constructs.

Status of bioreactors in the development of *in vitro* models

Bioreactors are utilised in a range of applications, from being used in the production of biopharmaceuticals to applications in tissue engineering such as cell expansion, generation of 3D tissue constructs and direct organ support devices [16] (see Table 1). Unlike 2D static culture, bioreactors provide a controllable environment in terms of pH, temperature, nutrient supply and shear stress for any cells or cellular constructs incorporated into them. However key limitations in 3D culture such as mass transfer of oxygen and nutrients to cells still apply. For example, cardiac muscle tissue contains a high density of cells and has a high oxygen demand which *in vivo* is supplied by a dense network of capillaries through the tissue. To gain a better understanding of the native environment of cardiac tissue and its influence on the synchronously contracting cardiac constructs, a

bioreactor-based model has been developed [17]. Incorporated into this device are rodent cardiomyocytes and fibroblasts grown on scaffolds with a parallel array of perfused channels to mimic the capillary network. Blood was mimicked by supplemented media and the model used to assess a number of parameters such as the impact of perfusion rates of oxygen on viable cell density to the define the conditions needed to culture cardiac constructs with clinically relevant thickness [18].

Hollow fibre and perfusion systems in particular are widely used in the generation of *in vitro* models. In addition to allowing users to investigate the impact of environmental conditions on cells, they can also be used to model pharmacological interactions of drugs with cells in infectious states [19] or model disease states [20].

Progression from these systems has generated multi-compartmental bioreactors that mimic the physiological environment more closely. As well as being used clinically [21], they are being utilised as *in vitro* models, like the scaling down of a 3D perfusion multi-compartment hollow fibre liver bioreactor for use in *in vitro* pharmacological studies [22]. This bioreactor consists of three interwoven sets of hollow fibre, two for counter current medium perfusion and one for gas supply (Fig 1). Primary human liver cells (both parenchymal and non-parenchymal) are co-cultured in the extra capillary space (ECS), and have been shown to exhibit prolonged CYP enzyme activity when maintained for over 3 weeks, enabling long term pharmacological studies to be carried out. Hollow fibre bioreactor based models are also being developed for the blood- brain barrier (BBB) and kidney. It is important to critically assess the individual features of established systems so that optimised models for pharmaceutical research can be generated, and this will be the focus of the remainder of the review.

Perspectives from established systems used within pharmaceutical research

Bioartificial Liver (BAL) devices

The liver is the main excretory organ within the body, but as well as eliminating waste products through the bile, it carries out a variety of highly specialised functions; oxidative detoxification, intermediate metabolism and supply of nutrients, modulation of immune and hormonal systems and protein and macromolecule synthesis [6]. Disruption in these functions due to liver disorders such as acute or chronic liver failure can lead to a possible 90% mortality rate if essential hepatic functions are not restored during the critical phase of liver failure [23]. Therefore, there is great emphasis on the development of reliable *in vitro* models to assess drug interactions and pharmacological responses to prevent drug induced acute liver toxicity, and to accurately model liver disease states for the development of NCEs targeting disease induced liver failure.

Bioartificial liver (BAL) devices have gone through many iterations whilst being developed as an extracorporeal device for the treatment of liver failure [24]. Early flat plate and monolayer bioreactors exhibited uniform cell distributions and microenvironments, but presented complex scale up issues and a low surface area to volume ratio [25] [26]. Perfused beds/scaffolds promote 3D architecture of seeded cells, which when considering the complex architecture of the liver, is an immense advantage. The cells showed improved metabolic and synthetic function compared to cells cultured in a spinner flask [27], however, exposure to shear forces and non-uniform perfusion of medium require an improved bioreactor design. Conventional hollow fibre cartridges offer increased surface area for attachment and immunoisolation of cells when seeded in the ECS [28]. Cells are also protected from shear as medium flows through the fibres. However non-uniform cell distribution along the fibres is a common problem, and although being beneficial in terms of support and improved cell growth and performance if adapted to the specific cell type [29], membranes can present a physical barrier against the transport of nutrients and metabolites. As mentioned previously, development from standard hollow fibre bioreactors to multi-compartmental devices such as that in Fig 1 has been a natural progression considering the native architecture of the liver and has been utilised for both clinical and pharmacological applications [22].

Cell Sources

Hepatocytes are the primary choice for use in BAL systems as they are recognized to be the closest cellular model to the liver [30] [31], and carry out most of the *in vivo* metabolic processes including the synthesis and excretion of albumin, metabolism of amino acids, urea production and the processing and elimination of drugs and toxins. Phase I and phase II drug metabolising enzymes are crucial in the processing of drugs within the liver, so preservation of these enzymes within a model is essential to reproduce cellular environments *in vivo*.

Cell lines have frequently been utilised for *in vitro* studies due to prolonged cell survival and ease of maintenance compared to primary hepatocytes. Popular liver derived cell lines such as the HepG2/C3A cell line derived from hepatomas have been used in pharmacological studies, recently being utilised in microfluidic bioreactors to examine the sensitivity of the models in response to various xenotoxins [32] [33]. However, compared to primary hepatocytes, functionality is often lost or altered in these cell lines [6]. For instance, phase I enzymes were expressed at significantly lower levels in HepG2 cells relative to primary human hepatocytes [34]. Therefore, primary cells would be the ideal for incorporation into a pharmacologically relevant *in vitro* model. However, recent work has demonstrated the successful incorporation of a HepaRG human hepatoma cell line into a 3D BAL device, in which metabolic and transporter functionalities of the cells are preserved from a mere few hours in suspension to at least one week in the 3D device, thereby enabling performance of longer-term *in vitro* pharmacological studies[35] [36] [37].

Primary porcine hepatocytes have been widely incorporated into BAL devices [38] [39] as they are readily available, are able to maintain differentiated metabolic functions under certain culture conditions, provide a high yield of cells [40] and have a similar metabolic profile to human hepatocytes [41]. The environmental triggers that sustain differentiated hepatic function *in vitro* however, have not been fully characterised [42].Utilising porcine cells addresses the problem of finding a sustainable source of hepatocytes; however, it would be important to obtain cells derived from a human source to establish a representative model of the natural physiological environment for *in vitro* pharmacological studies in both normal and diseased states.

Primary human adult hepatocytes are considered to be the gold standard for biocompatibility and functionality within BAL systems [6] [43], but exhibit a rapid, time-dependent, general loss of function *in vitro* [7]; including reduced cytochrome P450 and other biotransformation enzyme activities [44] and the down regulation of liver enriched transcription factors such as CCAAT/enhancer binding protein alpha [43]. However, the use of complex chemically defined media has allowed hepatocytes to go through a number of cycles in culture as well as stabilize hepatocyte morphology, survival, and liver-specific functions [45] [38] [46] [43]. Co-culture with non-parenchymal cells has also shown to preserve liver specific functions including initiating physiological reorganisation of cells and

the distribution of canalicular transporters similar to the pattern found *in vivo* [22]. The mechanisms behind these changes are not fully understood, but highly conserved signalling pathways are thought to be involved [9].

There is also limited availability of adult human hepatocytes due to their acquisition from liver biopsies, which has presented problems with utilising this source in clinically used devices as the number of cells needed for seeding a BAL for the treatment of hepatic failure is quite large, approximately 2×10^{10} [26] [47]. However the number of cells needed for bioreactor based models are approx. 3×10^{8} per device [22], which could be attained with minimal culture *in vitro*.

Device designs and functionality

Within the native liver, hepatocytes are ordered in a 3D lobular network of hexagonal constructs, surrounding a central blood vessel. Individual cells possess polarity between the apical domain interfacing with bile canaliculi and a basal domain interfacing with the sinusoid, helping localise specific functions. During culture, hepatocytes flatten and spread upon attachment, whether grown on a flat surface or a scaffold. Although cell-cell junctions can be formed, the cytoskeleton is largely disrupted, and leads to loss of cell polarity, and ultimately liver specific functions [6].

Nonetheless, C3A cells and primary porcine cells have been utilised within hollow fibre bioreactors (HFBs) in clinically tested devices. The Extracorporeal Liver Assist Device (ELAD) consists of a dual pump dialysis system connected to one or more hollow fibre cartridges seeded with C3A cells in the ECS. Blood is ultrafiltered and pumped through fibres, with plasma flowing through the ECS. This direct interaction allows cellular processes to occur before the ultrafiltrate is filtered by a dual membrane to remove cells and cellular debris and returned back into the blood stream [48].

The liver has an oxygen rich environment, fed by the hepatic arteries and portal vein. Unsurprisingly, an improvement in functionality of C3A cells in HFBs was noted when red blood cells were added in culture medium [49], highlighting that an adequate oxygen supply is a prerequisite for the maintenance of hepatocytes *in vitro*. Considering this, the Excorp medical bioartificial liver support system (BLSS) incorporates an oxygenator and heat exchanger along with a blood pump and HFB seeded with primary porcine hepatocytes in the ECS [50]. Whole blood flows through the fibre, protecting the cells from host immunological responses [39].

Another BAL, the modular extracorporeal liver support system (MELS) has attempted to take into account the biliary system of the liver which excretes conjugated bilirubin and metabolites. Hollow fibre bundles, seeded with human hepatocytes from discarded donor livers are incorporated alongside a detoxification module for removing albumin bound

toxins. This provides an inlet of plasma and contact with hepatocytes in the ECS, an outlet of plasma, and hydrophobic membrane bundles for gas exchange inside the bioreactor [51]. The structure of the seeded bioreactor module is an early iteration of the multi-compartmental bioreactor being developed for *in vitro* pharmacological studies by *Gerlach et al* (described above and in Fig 1).

These systems, although showing positive biochemical changes in patients and maintaining patients through to transplantation, show no significant effect on survival, and therefore can be assumed to be unsuitable replicates of the environment *in vivo*. Aspects such as co-culture of non-parenchymal and parenchymal cells, efficient oxygenation of cells and the presence of a biliary system for the removal of toxic protein bound substances and other metabolites should be taken forward in the generation of both efficient *in vitro* models, as has been demonstrated in the multi-compartmental liver bioreactor (Fig 1), and clinically relevant medical devices.

Use of BALs in DMPK studies

Bioreactor-based models are currently being integrated into the DMPK process, initially assessing the functionality of cells within the systems relative to drug transporter and metabolic enzyme expression and function. When investigating the metabolism of diclofenac, freshly isolated human hepatocytes were found to produce 90% less hydroxydiclofenac, the hydroxylated product of diclofenac generated directly by the cytochrome P450 enzyme CYP2C9, after a 3-day culture in 2D conditions compared to a high level of metabolites from human hepatocytes in the multi-compartmental bioreactor described above [35] [22]. The major intermediary diclofenac metabolite, diclofenac acyl glucuronide (produced from the glucuronidation of diclofenac by UDP-glucuronosyltransferase (UGT) enzymes before hydroxylation by the P450 enzyme CYP2C8), was found to be absent in human hepatocyte suspensions, but present in the hepatocyte seeded bioreactor, possibly due to the bioreactor system mimicking the *in vivo* environment and allowing the efflux of metabolites away from the cells, thereby avoiding further metabolism. Overall, the relative levels of the metabolites found in the suspensions versus the 3D cultures were similar; however, all human *in vivo* metabolites were detected in the bioreactor culture, whereas one major *in vivo* metabolite was not present in the hepatocyte suspension. In addition the biotransformation capacity of the hepatocytes within the bioreactor was retained for at least a week, providing a good model for metabolite investigations from slowly metabolised drugs [35]. The activity of CYP3A4 was demonstrated in the 3D bioreactor system by monitoring the metabolism of atorvastatin acid (ATA) to its 2- and 4- hyroxylated metabolites [36], and for HepaRG cells in the 3D system by monitoring midazolam, which indicated that CYP3A4 activity was retained for up to 2 weeks [48]. This retention of CYP3A4 activity is longer than that currently available in other *in vitro* models.

Transporter expression is also a key aspect in the functionality of hepatocytes for DMPK screens. Within the bioreactor, immunohistochemical staining of multidrug resistance associated protein (MRP2) and multidrug resistance protein (MDR1), both bile canicular efflux transporters, showed expression, localisation and distribution similar to native liver tissue for at least 2 weeks within the bioreactor [37] [22]. The basolateral uptake transporter organic anion transporting polypeptide 1B1 (OATP1B1) expression has also been investigated on hepatocytes within the 3D system being preserved for 9 days, Functional activity of OATP1B1 was demonstrated by the transport of substrates estradiol- 17β-D-glucuronide (E17βG) and ATA in the presence and absence of the inhibitor estrone-3-sulphate (E3S), and was retained for 7 days compared to the decrease of functional activity of OATP1B1/1B3 in plated primary human hepatocytes after just 2 hours [52] [36]. When assessing drug-drug interactions, OATP1B1 is a vital hepatic transporter to evaluate, as it is inhibited by many commonly used drugs, and is responsible for the targeted uptake of the widely prescribed statins to their site of action [53]. This has been demonstrated by similar increases in the elimination half-life of ATA in the presence and absence of OATP1B1 inhibitors, with a 2.7 fold increase in the presence of rifampin [54], and a 1.7 fold increase in the presence of E3S within the bioreactor model. The difference in these values had been attributed to experimental setup and potencies of the two inhibitors, and it is also been noted that the major limitation of this model lies in the variable quality of isolated human hepatocytes from different donors [36]. However, these initial studies into the functionality of drug transporters and metabolic enzymes clearly establish the potential of this model for use throughout the DMPK process.

For both the medical devices and *in vitro* models highlighted above there are currently no standardised criteria to define the efficiency and efficacy of these systems. Furthermore, any criteria applicable to a device will be partly dependent on the type of bioreactor used, i.e. hollow fibre systems require tight monolayers of cells on the extra luminal side of the fibre membrane. However, cellular metabolic functions and enzymatic and transporter retention is paramount in all bioartificial liver systems. From the clinically relevant devices mentioned above, there is little focus on the performance of cells, and factors used to determine the efficiency of the systems before being used in clinical studies are not clearly stated. Devices that have been utilised for pharmaceutical (DMPK) research go further in defining the criteria used to assess the models, such as monitoring metabolic activity via albumin synthesis, lactate dehydrogenase or aspartate aminotransferase, and drug metabolising enzymatic activity via administration of drugs with cytochrome P450-dependent metabolism, as well as drug transporter functionality and visualisation by inhibitor transport assays and staining [22] [35] [36] [37] [52] [53] [54]. With the progression of these systems, both medical and in vitro models, standardised criteria defining the performance relative to native conditions of the above mentioned factors should be produced.

Blood brain barrier models

The BBB is one of the most important lines of defence against infections in the brain, acting as a physical and metabolic barrier between the central nervous system (CNS) and systemic circulation and maintaining homeostasis within the brain. The capillary structure is distinct from those found in peripheral tissues with the presence of tight junction proteins between endothelial cells, the restriction of transcellular movement of molecules and contact of endothelial cells with astrocytes to provide support [55], protection from hypoxia and aglycemia and to separate capillaries from neurons [56].

The decreased paracellular permeability of the BBB, while possessing multidrug transporters such as P-glycoprotein, prohibits the transport of large drug molecules into the CNS, presenting the need for a reliable *in vitro* model for ADME studies. Existing models have not been characterised as well as liver devices, partly due to their lack of clinical relevance regarding the generation of an extracorporeal device, but also due to the complexity of cell alignments and functionality within the model. However, like the BAL devices, HFBs have been developed and utilised as 'dynamic' models of the BBB [57].

Model designs

Unlike liver models and bioartificial devices, there is no one cell type that can be incorporated to mimic the BBB. Most models reported in the literature use two cell types; endothelial cells and glial cells (astrocytes). Early models simply used mono/co-cultures of endothelial cells (EC) alone or with glial cells cultured on transwell plates. This approach is attractive due to its simplicity, however, primary ECs lose their BBB properties *in vitro* due to the absence of stimuli that are normally present *in vivo* [57]. Furthermore, specific transporters such as P-gp are down-regulated in the absence of astrocyte-derived soluble factors leading to abnormal permeability across the monolayer [58]. This de-differentiating process may also be accelerated by non-physiological conditions such as the presence of serum on both sides of the polarised EC monolayer instead of only on the luminal surface *in vivo*. Co-culture models incorporating glial cells mimic the *in vivo* situation more closely, demonstrated by the increased expression of brain endothelial marker enzymes, transporters, and an increased trans-endothelial electrical resistance (TEER) across the barrier indicative of a 'tight' monolayer. This model is effective for studies around the function of the BBB and processes and interactions between the ECs and the glia [57]. However, for a replicative model of the BBB, factors such as shear stress, which is vital to promote growth inhibition and differentiation of ECs and allows trafficking of metabolic fuels to the brain, should be incorporated into animal based models before ideally progressing into human based models to allow direct extrapolation of data from studies, and functional and physiological mimicry of the human BBB *in vivo* [59].

Dynamic (flow based) models incorporate HFBs with coated fibres seeded with ECs (intraluminally) and glial cells (extraluminally). In an early model developed by *Stanness et al* [60] ECs and corresponding glial cells from human, rat and bovine sources were cultured successfully in a HFB coated with ProNectin (Table 2) [60]. Morphology and growth patterns were consistent with all cell types, however it was noted that when differing types of ECs and glial cells were cultured, such as human umbilical cord ECs with C6 cell line glial cells, BBB properties were not developed. Poor EC growth in the absence of glial cells within the hollow fibres was observed via low transendothelial resistances and studies assessing the transport of morphine demonstrated permeability ratios to be similar to *in vivo*, but absolute values to be lower. This was thought to be due to the differences in flow rate patterns in the devices and from *in situ*. The results supported the use of HFBs in BBB models; however, as bovine aortic endothelial cells + C6 seeded cartridges were used for drug transport studies, utilising human brain ECs in the HFBs would be a natural progression.

Neuhaus et al [61] utilised a commercially available bioreactor from the same manufacturer as *Stanness et al*, and seeded immortalised porcine brain microvascular endothelial cells (PBMEC/C1-2) intraluminally with the glial cell line C6 cells in the extra capillary space. Medium was pumped through the system applying shear stresses of 2.7-3.9 dyne m^{-2}, mimicking conditions in brain capillaries. Results of cell growth and transport of benzodiazepenes were compared between the transwell and flow based models. They showed that PBMEC/C1-2 cells seeded on the transwell inserts started detaching after 5 days prohibiting the use of transwell systems in long term experiments, whereas growth and morphology of the ECs in the HFB was affected due to exposure to flow, prolonging the survival of the cells within the device. Permeability coefficients of benzodiazepenes were also shown to be lower in the HFB model, presumably due to tighter monolayers and improved barrier functions within the device [61].

The BBB is a very active site in terms of transport, expressing multiple drug transporters to restrict compounds with high affinity for efflux transporters and low transcellular membrane permeability into the CNS. However, due to its complexity, there are very few established *in vitro* models, and CNS targeted compound analysis relies heavily on *in vivo* and *in silico* assessments. P-gp (MDR1) is a predominant efflux transporter in the BBB, capable of limiting the penetration of a wide range of substrates into the brain. For this reason, MDCK-MDR1 cellular bidirectional transport assays are currently used in DMPK screening to assess both CNS penetrating and restricted compounds, as appropriate depending upon target profile [4]. Differences in transporter expression and passive permeability have been shown in 2D static co-culture models (described above). However, investigations have also highlighted interspecies variability, with a 2-fold higher permeability in a rat EC and glial model than in the human counterpart. The limitations of the current transwell model utilising transfected cell lines are its static nature and lack of availability of human cells. However, this system could be improved by using rat ECs and glial cells, to better represent the *in vivo* environment than is currently available with transfected cell

lines alone, in order to account for the difference in permeability [62]. Due to the nature of individual transporter testing in current 2D cultures, 3D models exhibiting BBB characteristics could also be utilised for drug-drug interaction studies as the 3D liver models are starting to investigate.

Overall, the outcomes of these models are promising for the use of HFBs in pharmacological applications. However, the human brain is more complex than its rodent counterpart, and although many drug transporter proteins are expressed in rodent ECs [57], primary human ECs and glial cells would ideally be incorporated into an optimised device. This may be difficult to generate for large scale studies as healthy human glial and ECs are scarcely available, so one possible source would be the differentiation of stem cells into these cell types. However, extensive work in stem cell differentiation and characterisation from embryonic and induced pluripotent stem cells is needed, and is not a viable source for models at present. Recreating aspects of the microenvironment of the CNS in terms of ECM proteins surrounding glial cells may also improve performance of the cells within the device.

Progressions in bioartificial kidney (BAK) devices as an *in* vitro model

The need for an optimised model from existing clinical devices

The kidneys are primarily responsible for the excretion of waste products such as urea and ammonia from the body through the urine. They also provide a variety of significant secondary functions through homeostatic, endocrine, immune and metabolic processes.

Chronic kidney disease (CKD) is a growing problem within the UK, with around 1.8 million people diagnosed in England alone. This is putting a growing burden on the NHS, costing over £1.4 billion a year which is largely due to renal replacement therapy e.g. dialysis,

which showed a 29% increase in usage from 2002-2008, and complications such as heart disease and stroke [63]. This highlights the need for early diagnosis of the disease, more efficient and cost effective renal replacement therapies, more effective drug treatments and consequently, an optimised kidney model for pharmacological studies.

Existing bioartificial kidney (BAK) devices have been developed primarily as a renal replacement therapy. They incorporate both mechanical and biological processes, pumping haemofiltered blood through a renal assist device (RAD). Coined by *Humes et al* this device contains renal cells seeded in the lumen of hollow fibres of a standard haemofiltration cartridge [64], contrary to BALs which utilise cells within the ECS. Unlike dialysis, which just provides intermittent filtration, *in vitro* studies of the BAK device show differentiated active transport of essential nutrients such as glucose, amino acids and sodium bicarbonate, sufficient metabolic activity and important endocrine processes [65]. Although the current bioartificial systems have been utilised for clinical use, there is scope as with the systems mentioned previously to use them as a foundation for many other applications.

There have been two main groups focusing on the development of a BAK device for clinical use. *Humes et al* developed a device to treat patients with acute renal failure and multiple organ failure with a degree of success, while *Saito et al* focused on the prevention and treatment of long term complications in patients on maintenance dialysis [66]. Both devices however utilise the same bioartificial component, hollow fibre modules seeded with proximal tubule cells (PTCs), and pass ultrafiltered blood through the module before administering back into the patient (Fig 2). *Humes et al* [67] utilised commercially available haemofiltration cartridges, with polysulfone (PSF) based fibres seeded with human PTCs (hPTCs). Although confluent monolayers of cells were observed within the fibres [65], *Saito et al*, concurring with *Zink et al* [68] has commented on the lack of biocompatibility of PSF and PSF-based membranes for generating a confluent monolayer of cells [66]. The clinical trial undertaken with the BAK device from *Humes's* group has also been critically assessed, noting the lack of documentation on expected effect size, the incompletion of treatment of patients assigned to CVVH + RAD treatment and the lack of statistical significance of the primary results with comparisons performed as an as-treated rather than an as-randomised intention to treat sample [69].

Cell Sources

To create an efficient, reliable model of the kidney *in situ*, a BAK device must incorporate a cell type that can carry out a number of native functions, and so research has been drawn to renal PTCs. These cells perform a variety of renal specific functions including reabsorption, metabolic and transport functions, the secretion of uremic toxins and xenobiotics and performing immunomodulatory functions [70]. Renal PTCs also possess a range of transporters such as organic anion and organic cation transporters for basolateral

drug uptake and ABC transporters present on the apical surface for luminal excretion into urine. Many of the transporters are polyspecific i.e. accept compounds of different sizes and molecular structures, and have overlapping substrate specificities [71].

Primary hPTCs are required for clinical BAK devices but the sheer number of cells needed (approximately 10^9 renal cells per device) is a major obstacle to their widespread use, driving the search for alternative cell sources. Human PTCs have also been used as a well-characterized *in vitro* model of the kidney in drug transport studies, owing to the classification of specific carrier proteins on the plasma membrane by substrate specificity and functional assessment, and the prediction of drug-drug interactions based on competitive inhibition for transport [72]. Two transporters, organic anion transporter (OAT) 1 and OAT3 are thought to be the major OATs responsible for the basolateral uptake of various organic ions from the blood *in vivo*; OAT3 action demonstrated by the mediation of rosuvastatin uptake *in vitro* in the presence and absence of probenecid, a uricosuric drug [73]. Probenecid has also been shown to be a potent inhibitor of OAT2 and OAT4, where $PGF_{2\alpha}$ uptake by OAT2, and estrone-3-sulfate uptake by apically located OAT4, was inhibited in the presence of probenecid. The main uptake transporter of organic cations in hPTCs is thought to be organic cation transporter 2 (OCT2) located on the basolateral membrane. Functionality has been demonstrated in hPTCs by the decrease in uptake of ASP^+ in the presence and absence of the inhibitor quinine [74]. Although drug-drug interactions for organic cations have been observed in other cell sources such as LLC-PK1 cells, little data on the drug interactions for OCT2 has been documented [75]. Similar to liver, P-gp is documented to transport a wide range of xenobiotics, in particular anti-cancer agents such as methotrexate and cisplatin [76] in hPTCs. However, in hydrophilic statin efflux it has been found to have a minor role compared to the MRP2 and BCRP efflux transporters [73]. Differences between effects of drugs *in vivo* and *in vitro* has also been highlighted by the inhibitory effects of KW-3902 and betamipron on OAT4 *in vitro*, which showed no significant inhibitory effects on OAT4 *in vivo* [77].

The expression of both phase I (11 CYP enzymes and 3 glutathione *S*-transferases (GSTs)) and phase II (3 UGT and variable expressions of 3 sulfotransferases) drug metabolising enzymes have also been found to be retained within primary hPTC cultures. Furthermore, when grown in a confluent monolayer, which is necessary in current BAK devices, the expression of these enzymes were generally maintained at a measurable level, albeit lower than in the native tissue [78]. Selective drug transporter loss upon primary cell culture has been identified to be a consequence of dedifferentiation. However, recent novel human tubular kidney cell cultures grown on permeable filter supports have been shown to express a wide range of drug transporters due to the co-culture of proximal and distal tubular cells [71]. This finding is concurrent with the increased function from co-culture of hepatocytes and non-parenchymal cells in BAL devices. Collectively, these results indicate that primary cultures of hPTCs can metabolize drugs, provide good models for drug interaction studies and benefit the clinical setting when incorporated into BAK devices.

Although hPTCs do not always represent *in vivo* functionality, these results could be improved by the incorporation into a dynamic bioreactor, providing a more accurate model of hPTCs *in situ*.

Using immortalized cell lines eliminates the problem of needing vast numbers of cells for studies. Methods such as cloning, derivation from cancerous cells and viral transfection can all produce genetically stable, but functionally altered cells. Human cells are relatively difficult to immortalize and do not express differentiated features *in vitro* [65][79]. The established cell line HK-2, generated from the infection of human PTCs with an immortalizing construct from the human papilloma virus, and the more recently developed human kidney proximal tubular cell line (HKC) from exposure of renal epithelial cells to Adeno 12:SV40 hybrid virus, therefore do not provide an adequate replacement for primary cells for use in clinical BAK devices [79]. Other established animal derived cell lines such as LLC-RK1, LLC-PK1 and MDCKII have also provided adequate models for research, but interpretation to the human situation is often difficult due to species differences, especially with regard to transporters and enzymes. Drug transport with human renal cell lines has not been widely researched, possibly due to the substantial loss of differentiated function from the immortalization of human cells, and primary hPTCs or transfected animal cell lines such as MDCKII-MDR1 are more frequently used. This highlights the challenge of generating a physiologically complex, well characterised and validated *in vitro* cellular model which can replace current FDA recommended cell transport test systems such as P-gp transfected MDCK or LLC-PK1 cells [80].

Porcine PTCs are widely used in kidney research due to their reliability, anatomical and physiological similarity to hPTCs. In preclinical studies, porcine PTCs in a BAK device were shown to tolerate the uremic environment while providing metabolic, reabsorptive and endocrinologic activity in uremic dogs [81], allowing research to further investigate the benefits of BAKs, and progress to hPTC utilization for clinical trials. There are few studies into differences in drug interactions and metabolism between porcine and human PTCs to date, although drug transporter expression on primary porcine PTCs and cell lines are comparative to native human kidney cells (Table 3). Both primary and porcine cell lines are readily available and are an established model for drug toxicity, however, an optimized model for drug toxicity would require human PTCs instead of the readily available established porcine model.

Due to their multipotency and self-renewal capabilities, embryonic stem cells may provide a reliable source of PTCs for use in BAK devices when exposed to the right environmental cues [64]. Studies into human ESCs differentiating into renal cells have not been widely undertaken, however preliminary studies have shown integration of undifferentiated and mesoderm- derived ESCs into the stroma and developing nephrons [82]. They have also been shown to express a wide range of early renal markers, and exhibit up-regulation of kidney precursor markers such as EYA1, LIM1, CD24 [83]. The generation of

the different types of renal cells from human ESCs however still needs to be thoroughly investigated [82]. Due to the sparse studies and various obstacles, drug metabolism and transport by human ESC-derived renal cells has not been documented, but will be essential in any progression of stem cell utilisation towards potential *in vitro* cell models used in pharmaceutical research.

Device designs

As mentioned above, the existing devices used for clinical applications utilise haemofiltration cartridges seeded with hPTCs. However for an *in vitro* model, device design could be optimised. For example the number of cells needed could be greatly decreased due to the reduction of the size of the cartridge. Hollow fibre cartridges could also be greatly optimised, as commercially available modules have varying degrees of biocompatibility. Tailoring fibre materials to different cell types and sources have been thought to improve cell performance, and for primary hPTCs it is thought that some of the membrane properties needed include a hydrophilic, negatively charged adhesive surface [29]. When a variety of commercially used membranes, such as regenerated cellulose (RC), PSF/PVP and polyethersulfone (PES)/PVP coated with ECM, were tested with primary hPTCs, performance of the cells was not improved, indicating that these materials are not suitable for use in BAK devices. The differing properties between cell sources is further highlighted by reports that PSF membranes coated with ECM are able to maintain both primary porcine [84] and LLC-PK1 cell monolayers for 3 weeks after confluency [85].

Coating commercially available fibres with tailored ECM is preferable to tailoring membrane materials due to the ease of assembly and cost effectiveness. Various single and double coatings have been tested, including collagen IV and laminin blends with a variety of growth factors [70]. Single coatings demonstrated little improvement on hPTC performance within the fibre however double coatings of DOPA (3,4-Dihydroxy-Lphenylalanine) and collagen IV on haemocompatible membranes have revealed functional epithelia formed by hPTCs in a HFB [68]. Bioreactor configuration should also be taken into consideration to achieve both native mimicry and produce an optimal environment for the cells to proliferate. Previous bioartificial devices utilising hollow fibre cartridges have encountered problems with the non-uniform distribution of cells and the lack of transport of gases and nutrients [86], so including an oxygenator or aerated medium would need to be considered. Additionally, in order to mimic the kidney the device would need to include a tubule (hollow fibre) and capillary/blood (extracapillary space) section with shear rates according to native conditions. These would be higher in the ECS (typically 10 dyn/cm² on endothelial cells *in vivo*) than in the fibre (typically 1 dyn/cm² on PTCs *in vivo*) [87]. This may lead to movement of molecules through the membrane if the monolayer of cells is not tight, and affect cell performance due to varying pressures. However, from previous devices used clincally, no

loss of membrane integrity has been reported when using different flow rates in the tubule and ECS [67].

Emerging *in vitro* models have also adhered to the established hollow fibre structure. The Wyss Institute at Harvard University have introduced 'organs on a chip'. These microfluidic devices, in this case 'kidney on a chip' utilise hPTCs seeded within a microfluidic channel apposed to a polyester membrane. The channel is immersed in medium, and a shear stress of 0.2 dyn/cm^3 is applied through the channel (Fig 3). Cytoskeletal proteins, tight junction proteins, ion transporters and specific drug transporters (e.g. OCT2) have shown improved results in a fluidic model compared to a static version, and the nephrotoxic compounds such as cisplatin have shown the ability of the microsystem to recapitulate the *in vivo* toxicity of the drugs. Overall, the system presents a substantial answer to the need of a reliable 3D *in vitro* model [88]. However, functional characterisation of the drug transporters within the device that are acknowledged to be of clinical importance to drug disposition by the FDA, namely P-gp, BCRP, OCT2, OAT1 and OAT3, should be undertaken to establish an ideal model system [89].

Conclusion and future perspectives

Bioreactors have been utilised in many different areas of industry and research, and for the progression of *in vitro* models of 2D cultures and suspensions to 3D constructs mimicking the natural physiological state *in situ*, they have proven to be crucial. The generation of bioartificial devices provide both the structural and biological aspects, which have been exploited for clinical and research purposes. The most established bioartificial system, the bioartificial liver, is a prime example of the development of a bioreactor designed initially for therapeutic use, being utilised as an *in vitro* model. From simple perfused bed and hollow fibre bioreactors, focus on the native liver structure and cellular functionality within the device has prompted multi-fibre bundle designs.

In addition to bioreactor design, development of the BBB models have highlighted the importance of cell sources, especially with co-culture of different cell types, within a fluidic system opposed to a static culture. It is understood that primary human cells are the

gold standard for an *in vitro* model, however, due to the difficulty of acquisition, BBB models have had to utilise other primary sources. Embryonic stem cells could be a possible answer to the dilemma of availability for the future; however, more research is needed to investigate the difference in properties between primitive and mature human CNS and endothelial cells before incorporation into a device for DMPK investigation.

Bioartificial kidney devices have followed the progression of BAL systems. From development for clinical use as a renal replacement therapy for acute and chronic renal conditions, they are now starting to reveal their potential as *in vitro* models (Fig 4). Primary human cells have been utilised within the system, and are fairly easy to acquire and maintain, especially with the reduced cell number requirement in a model. Co-culture with distal tubule cells however has not been fully investigated within a BAK device, and functional characterisation of drug transporters crucial for ADME studies have not been undertaken in the existing microfluidic system.

To date, there are no universal criteria laid out to aid the development of an acceptable/suitable bioreactor. However, it is widely accepted that *in vivo* micro environmental aspects such as cell viability, cell-cell interactions, cell-matrix interactions, tissue architecture and cell oxygenation are vital to give an accurate representation of the physiological state, and consequently the effects of new chemical entities [3]. It is therefore important to bear in mind those factors which are specific to the desired tissue when developing *in vitro* models for use in pharmaceutical research. Furthermore, it is noticeable that little or no measurements are currently taken from these bioreactor-based devices to help control or monitor cell health or functionality during use. We thus anticipate that, with the continuing advances being made in sensor technology, the next generation of devices will incorporate these to either improve device function or longevity.

Bioartificial systems have started to show great promise in the generation of *in vitro* models for various organs and disease states. Observations from existing systems are the importance of mimicry of both structure and environments *in situ* in the bioreactor, the choice of cell and source to model the functions of the organ and essential factors for maintaining cellular function such as oxygen availability and nutrient and metabolite delivery. With these factors in mind, successful models utilising bioreactors can be achieved in the near future and will hopefully provide a better means to predict clinical outcome from *in vitro* data.

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| Bioreactor type	Pros	Cons	Uses	Ref

Figure legends:

Figure 1: (a) Smallest capillary unit with 2 medium capillaries that are independently perfused (red and blue) and one gas capillary (yellow); cells are cultured within the extra-capillary space (cell compartment). **(b)** The bioreactor consists of 3 interwoven capillary bundles each made of multiple hollow fibre capillaries for counter-current medium perfusion (red and blue) and gas supply (yellow), which allows decentralised nutrient and O_2/ CO_2 exchange with low gradients. **(c)** Down-scaling of the clinical- scale bioreactor prototype with a cell compartment volume of 800ml was realised by reducing the length and number of capillary layers in 2 axes of 3D space within the bioreactor, resulting in a laboratory- scale bioreactor with a cell compartment volume of 8 ml and a further down-scaled model with a cell compartment volume of 2ml. Figure and caption taken from *Zeilinger et al* [22]

Figure 2: Schematic diagram of **(a)** bioartificial kidney device used for the treatment of acute renal failure developed by *Humes et al* **(b)** bioartificial kidney device developed as a renal replacement therapy for chronic renal failure by *Saito et al*. Based on device layouts from [67], [66]. The devices have similar components and configurations; however the device by *Saito et al* incorporates a continuous haemofilter for continuous treatment.

Figure 3: Schematic of human kidney proximal tubule-on-a-chip in the form of multi-layer microfluidic device by integrating a polydimethylsiloxane (PDMS) microfluidic channel, a porous membrane, and a PDMS reservoir. Human primary proximal tubule cells were cultured on the device. Modified with permission from *Jin Jang et al* [88].

Figure 4: Key aspects from the progression of bioreactor based *in vitro* models and future developments [22] [61] [68].

Spinner flasks/ Stir tank
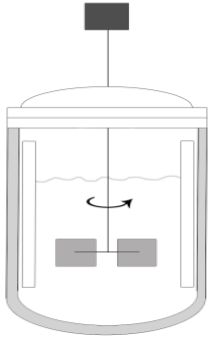
Pros:

- Induces

movement of oxygen and nutrient within culture medium
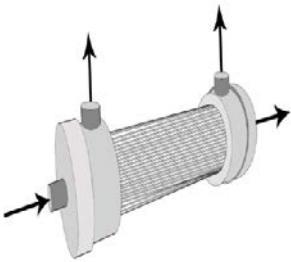
- Easy to scale up

Cons:

- Perception that shear stress can have damaging effects on cells

- Poor mass transfer when applying to static scaffolds

Uses:

Growth of cartilage constructs

[19]

Hollow fibre

Pros:

- Efficient surface area for attachment

- Potential for immunoisolation

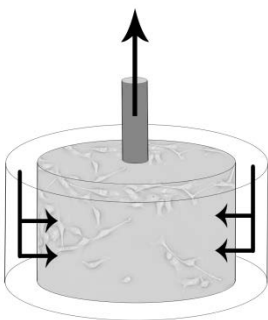- Perception that cells are protected from shear

Cons:

- Non-uniform cell distribution

- Fibre membrane can act as a physical transport barrier

Uses:

Generation of a bioartificial liver support system

[20]

Perfused beds/ Scaffolds

Pros:

- Uniform cell distribution

- Good distribution of nutrients and gas transfer
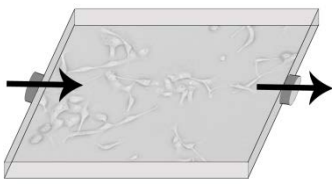
- Stable microenvironment

Cons:

- Complex scale-up

- Cells exposed to shear stresses

- Low surface area to volume ratio

Uses:

Cartilage construct engineering from chondrocyte seeded scaffolds

[21]

Flat plate + monolayer	Pros:	Cons:	Uses:	[22]
	- Promotes 3D structure formation	- Non-uniform distribution of cells	Early bioartificial liver system studies	
	- Reduced physical transport barriers	- Cells exposed to shear stresses		
	- Easy to scale up	- Nutrient and oxygen transfer issues		

Table 1: Bioreactor designs and uses in tissue engineering applications

Table 2: Performance of various cell types within the HFB in the generation of an *in vitro* BBB model

Endothelial Cell (EC)	Glial Cell	Days prolonged in culture	Ref
Rat brain microvascular endothelial cells (RBMEC)	Rat brain astrocytes (RBA)	30	[63]
Human brain endothelial cells	Human foetal astrocytes	Not reported	[63]
Bovine aortic endothelial cells	C6 glial cell line	30	[63]

Table 3: Drug transporter orthologues present on MDCKII and LLC-PK1 cell lines and primary porcine PTCs[1]

Transporter Family	Gene Product	Location	MDCK II	LLC-PK1	Primary Porcine Cells	Ref
Organic anion transporters	OAT1 (SLC22A6)	Basolateral	X	X	~	[85] [86] [87] [88]
	OAT2 (SLC22A7)	Basolateral				[85] [86]
	OAT3 (SLC22A8)	Basolateral	X	X	√	[85] [86] [87] [88]
	OATP1A2 (SLCO1A2)	Apical	X	X	+/-	[88] [89]
ATP binding cassette (ABC) transporters	MRP1 (ABCC1)	Basolateral	√	√	√	[85] [86] [87] [88] [89]
	MRP5 (ABCC5)					[85] [86] [87]
	MRP2 (ABCC2)	Apical	√	√	√	[85] [86] [87] [88] [89]
	MRP4 (ABCC4)					[85] [86] [87]
	MRP3 (ABCC3)	Basolateral	√			[85] [86]
	MRP6 (ABCC6)	Basolateral	√			[85] [86]
	MDR1 (ABCB1)	Apical	√	√	√	[85] [86] [87] [89]
	BCRP (ABCG2)	Apical	√	√	X	[85]
Organic cation transporters	OCT1 (SLC22A1)	Basolateral	X	X	~	[85] [86] [87]

Category	Transporter	Location				References
	OCT2 (SLC22A2)	Basolateral				[85] [86] [87]
	OCT3 (SLC22A3)	Basolateral				[85] [86] [87]
Carnitine/organic cation transporters	OCTN1 (SLC22A4)	Apical				[85] [86] [87]
	OCTN2 (SLC22A5)	Apical				[85] [86] [87]
Organic anion transporting polypeptides	OATP4C1 (SLCO4C1)	Basolateral				[87] [89]
Peptide transporters	PEPT1 (SLC5A1)	Apical	X	√	√	[85] [86] [87] [88]
	PEPT2 (SLC5A2)	Apical	X	X	√	[85] [86] [87]
Multidrug and toxic compound extrusion transporters	MATE1 (SLC47A1)	Apical				[87]
	MATE2-K (SLC47A2)	Apical				[87]

[1] Human drug transporter orthologues present on the cell lines and primary cells. Blank fields indicate no presence of transporters on the cells. Abbreviations: √, present; X, absent; +/-, only detectable after 35 cycles of PCR; ~, only present in freshly isolated samples.
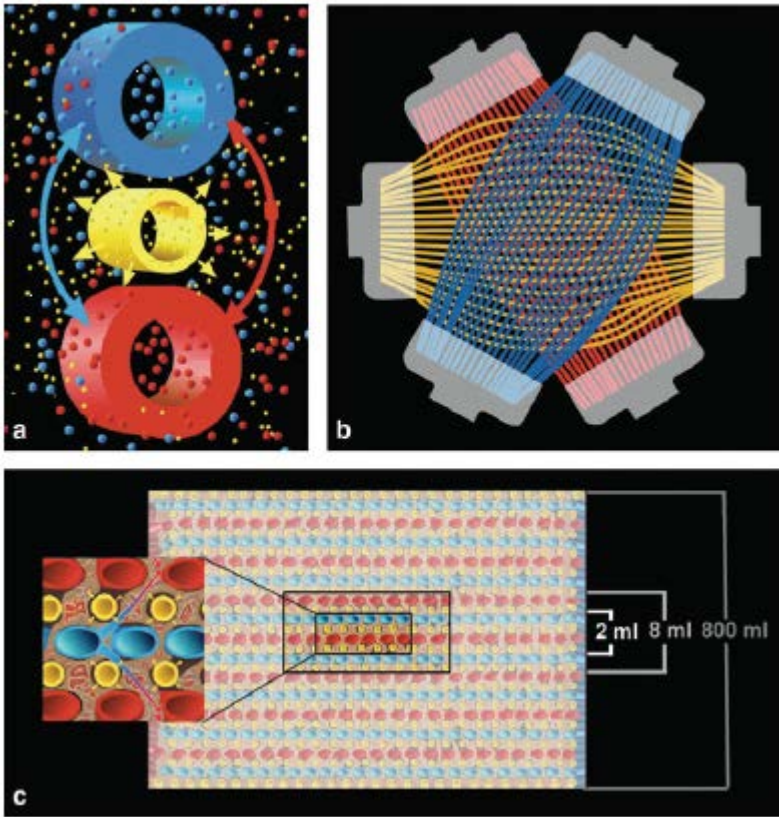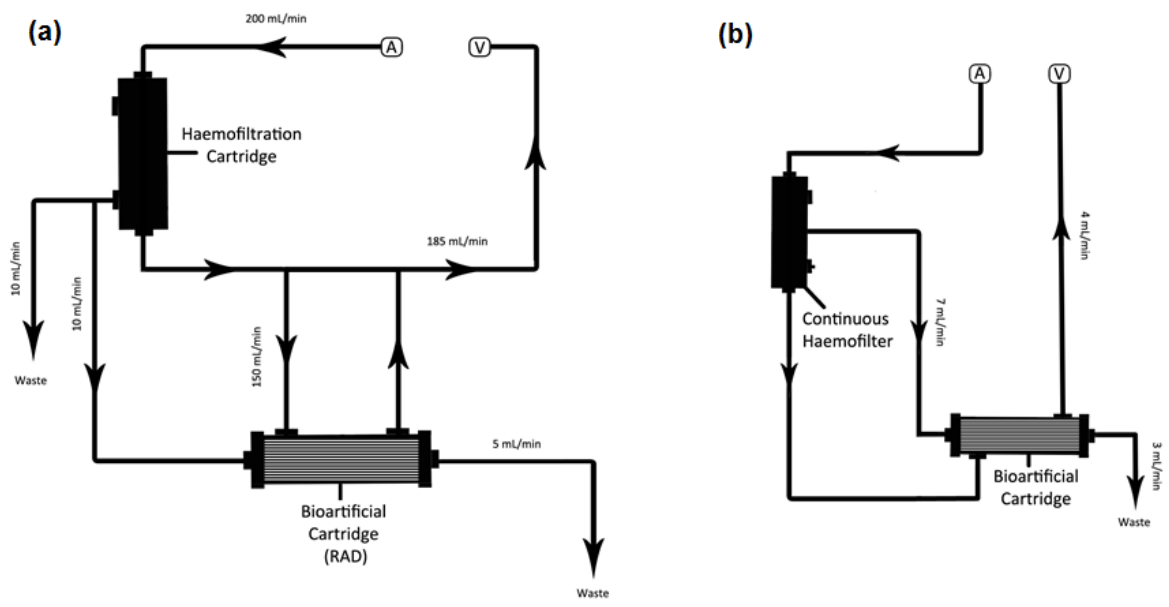
Fig 1.

(a)
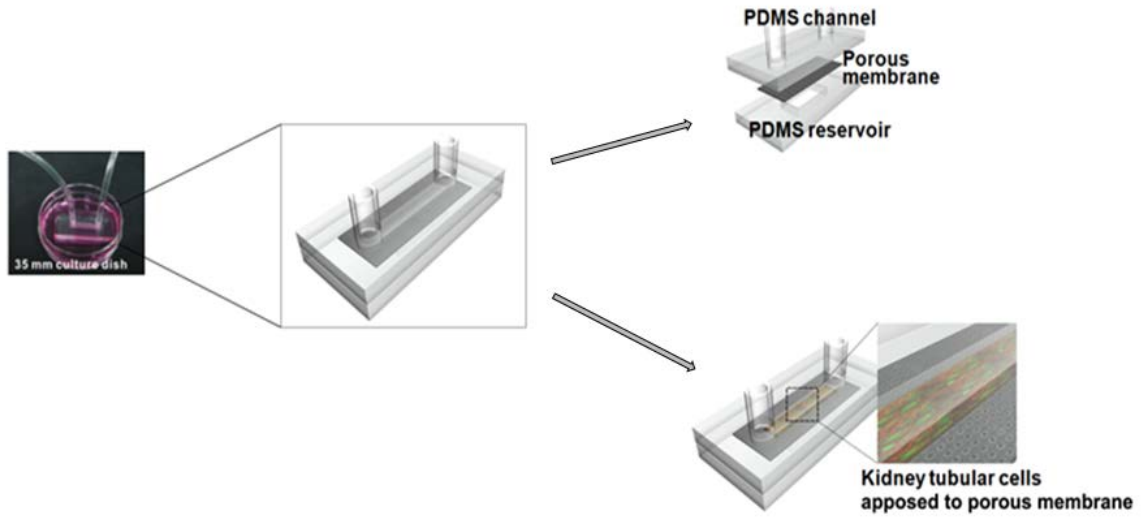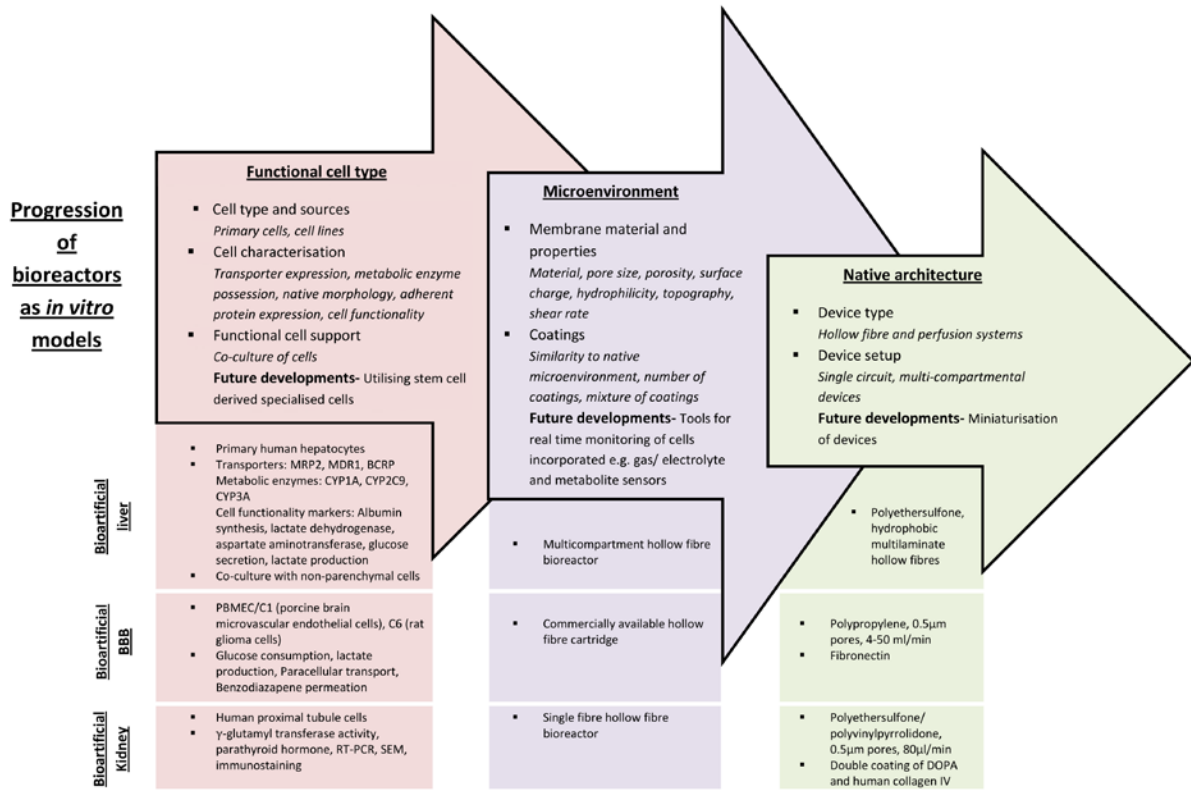
200 mL/min (A) (V)

Haemofiltration Cartridge

10 mL/min → Waste

10 mL/min

185 mL/min

150 mL/min

Bioartificial Cartridge (RAD)

5 mL/min → Waste

(b)

(A) (V)

Continuous Haemofilter

7 mL/min

4 mL/min

Bioartificial Cartridge

3 mL/min → Waste

Fig 2.

Fig 3.

Fig. 4