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Flow–perfusion bioreactor system for engineered breast cancer surrogates to be used in preclinical testing

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Abstract

There is a need for preclinical testing systems that predict the efficacy, safety and pharmacokinetics of cancer therapies better than existing *in vitro* and *in vivo* animal models. An approach to the development of predictive *in vitro* systems is to more closely recapitulate the cellular and spatial complexity of human cancers. One limitation of using current *in vitro* systems to model cancers is the lack of an appropriately large volume to accommodate the development of this complexity over time. To address this limitation, we have designed and constructed a novel flow–perfusion bioreactor system that can support large-volume, engineered tissue comprised of multicellular cancer surrogates by modifying current microfluidic devices. Key features of this technology are a three-dimensional (3D) volume (1.2 cm³) that has greater tissue thickness than is utilized in existing microfluidic systems and the ability to perfuse the volume, enabling the development of realistic tumour geometry. The constructs were fabricated by infiltrating porous carbon foams with an extracellular matrix (ECM) hydrogel and engineering through-microchannels. The carbon foam structurally supported the hydrogel and microchannel patency for up to 161 h. The ECM hydrogel was shown to adhere to the carbon foam and polydimethylsiloxane flow chamber, which housed the hydrogel–foam construct, when surfaces were coated with glutaraldehyde (carbon foam) and nitric acid (polydimethyl-siloxane). Additionally, the viability of breast cancer cells and fibroblasts was higher in the presence of perfused microchannels in comparison to similar preparations without microchannels or perfusion. Therefore, the flow–perfusion bioreactor system supports cell viability in volume and stromal contexts that are physiologically-relevant.

Keywords

bioreactor; 3D breast cancer model; stroma; perfusion; microchannels

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Conflict of Interest

The authors declare no conflicts of interest.

Ethics statement

No animal or human subjects were used in this study and no institutional ethical approval was required.

1. Introduction

Three-dimensional (3D) multicellular cultures perfused in microfluidic systems are promising technologies for drug development (Maguire *et al.*, 2009). The importance of these systems is underscored by recent participation of the US National Institutes of Health (NIH), the Defense Advanced Research Projects Agency, the Food and Drug Administration (FDA), universities and biotechnology industries in their development. Rapid, accurate and inexpensive assessment of drug efficacy, toxicity and pharmacokinetics prior to first-in-human studies is the primary goal of these systems. These efforts are motivated by the need to extend traditional two-dimensional (2D) cell culture systems into 3D environments that better replicate the microphysiological environment of breast cancer and more accurately predict drug efficacy and toxicity (Li *et al.*, 2010; Sharma *et al.*, 2010). Herein, we describe the development of a novel 3D flow–perfusion bioreactor system to support the tissue engineering of a multicellular breast cancer surrogate that would ultimately serve as a drug development platform for cancer therapeutics. Two key features of this technology are a 3D volume larger than that utilized in existing microfluidic systems and the ability to perfuse this volume.

Lab-on-a-chip microfluidic devices, a type of flow–perfusion bioreactor system, have recently been employed to study the relationship between cell types, extracellular matrix (ECM), diffusion and shear stress (Buchanan *et al.*, 2014; Park *et al.*, 2010; Zheng *et al.*, 2012). Key features of the lab-on-a-chip devices are a transparent support structure, enabling fluorescence and confocal imaging of fluid flow and cellular phenotypes as well as precise control over the geometry of flow microchannels (Barrila *et al.*, 2010; Hielscher and Gerecht, 2012). These devices are easily fabricated using soft lithography, require small volumes of medium for perfusion and facilitate parallel experimentation. Moreover, these lab-on-a-chip perfusion systems allow for insertion of only small volumes of ECM hydrogels (e.g. collagen) as substrates for epithelial cell growth, while microchannel diameter and chip thickness typically do not exceed 100 and 500 μm , respectively. These factors make the lab-on-a-chip approach well suited for microscopic visualization of flow and cellular events (Park *et al.*, 2010; Zheng *et al.*, 2012).

Breast cancer tumour volume is an important consideration in accurate modelling of breast cancers. As an example, triple-negative breast cancers average 4.1 ± 2.7 cm in diameter (Chen *et al.*, 2007); thus, *in vitro* systems designed to replicate breast tumour biology require larger volumes than lab-on-a-chip systems allow. Thicker microphysiological perfusion systems within flow–perfusion bioreactors have recently demonstrated ease of visualization and maintenance of cellular phenotype in the presence of well-defined flow characteristics (Buchanan *et al.*, 2014). Buchanan showed that breast carcinoma cells embedded in rat tail collagen I and perfused through a central endothelialized channel (711 μm diameter) maintained their viability for up to 72 h of culture. Additionally, a study by Chen *et al.* (2010) showed that perfusion (800 $\times$ 90 μm rectangular channel) over alginate-encapsulated breast tumour cells led to cellular aggregation after 5 days of culture; these cultures were maintained for up to 2 weeks in a microfluidic device. However, many of the 3D *in vitro* models of breast cancer to date have relied upon cell spheroids in suspension or cell aggregation in ECM volumes that do not recapitulate relevant volumes of *in vivo* breast

tumours and/or lack relevant stromal components, such as fibroblasts (Petersen *et al.*, 2003; Shaw *et al.*, 2004). Most of the 3D models that have used greater, more relevant, solid volumes have not supported long-term viability of incorporated cells, presumably a result of inadequate perfusion of nutrients and oxygen.

In addition to the volume required for tumour growth, the structural organization of tumour epithelial and stromal cells (e.g. cancer-associated fibroblasts) within the ECM volume supports relevant gene expression and cellular phenotype and influences drug sensitivity (Astashkina *et al.*, 2012; Schanz *et al.*, 2010; Weigelt and Bissell, 2008). Since the viability of cells embedded in the 3D tumour construct is dependent upon the model thickness, and since oxygen diffusion distance is <100–200 μm , it is essential that perfusion is provided that will support tissue oxygenation (Gibot *et al.*, 2010; Hielscher and Gerecht, 2012; Rouwkema *et al.*, 2008; Schanz *et al.*, 2010; Sukmana, 2012) in biologically relevant 3D models.

The flow–perfusion bioreactor system described here is designed to support the viability of human breast cancer cells and breast fibroblasts in an *in vitro* 3D construct with a more physiologically relevant volume. The goal of this technology is to provide 3D, humanized, engineered breast tumour models to supplement *in vivo* animal studies, where interspecies differences in physiology, drug metabolism and mechanistic responses to disease clearly do not reflect safety and toxicity results observed in human clinical trials (Astashkina *et al.*, 2012; Barrila *et al.*, 2010; Gupta and Kuperwasser, 2004; Haycock, 2011; Schanz *et al.*, 2010).

2. Methods and materials

Our flow–perfusion bioreactor system was designed with a volume (>500 μm in any dimension) to support cancer cell aggregation, recapitulate physiological cell function, support long-term cell maintenance and growth and accurately model responses to external challenges (i.e. drugs). Important elements of our system are prefabricated microchannels for medium perfusion, a thickness of 2.4 mm, structural support for the ECM containing breast cancer cells and stromal fibroblasts, ease of handling and potential for multiplexing with other bioreactors.

2.1. 3D construct fabrication and measurement of carbon foam purity and microchannel size

The 3D constructs are composed of ECM proteins supported by an inert backbone and perforated with microchannels. Bovine collagen I (BD Biosciences; 3 mg/ml stock solution solubilized in 0.012 N HCl, 1.9 mg/ml final concentration) and growth factor-reduced Matrigel (BD Biosciences), in a 9:1 v/v ratio, were chosen as the ECM components to mimic the ECM in the breast cancer microenvironment (Lochter and Bissell, 1995). The low viscosity of the ECM necessitated a structure to support prefabricated microchannel patency and to minimize shrinkage of the ECM hydrogel. A rigid carbon foam [reticulated vitreous carbon (RVC), ERG Aerospace Corp. or Ultramet] was chosen, as it is stiff, autoclavable and highly porous (ca. 500 μm pore size) (Fig. 1A) (Pec *et al.*, 2010; Wickham *et al.*, 1983).

Energy-dispersive X-ray spectroscopy (EDS) with an EDAX detector (Ametek, SEM; Quanta FEG 650, FEI) was used to measure carbon purity.

Importantly, since the 3D constructs are thicker than the diffusion distance of oxygen (100–200 μm), microchannels serve as conduits for perfusion of oxygenated medium and thus enabled oxygen and nutrients from the medium to diffuse throughout the ECM hydrogels. Microchannels were formed by hand-insertion of Teflon-coated stainless steel rods through the carbon foam prior to injection of the liquid ECM hydrogel (Fig. 1B). Naked-eye visualization was used to evenly space four rods within each carbon foam, with a target of 1 mm centre-to-centre spacing; however, histological sectioning artifacts prevented verification of the rod spacing.

The ECM hydrogel solution contained 13.7% v/v Dulbecco's modified Eagle's medium (DMEM), 1.9 mg/ml bovine collagen I, 10% v/v 10 $\times$ DMEM, 10% v/v GFR Matrigel and 0.524 M sodium bicarbonate (Thermo Scientific). This final collagen concentration was chosen to maintain a balance between the matrix stiffness necessary to withstand the pressures of fluid flow and the ability of the cells to migrate through pores in the ECM hydrogel (Carey *et al.*, 2012). ECM hydrogel solution was injected into multiple voids within the carbon foam via a 1 ml syringe and 27G needle, until all voids were completely filled (approximately 0.2–0.3 ml/foam). Bubbles that formed during the injection process were removed via negative pressure with the syringe and needle. Polymerization occurred in the incubator (37°C, 5% CO₂) over 45 min. After ECM hydrogel polymerization the rods were removed, resulting in parallel microchannels 250 and 400 μm in diameter.

To measure the shrinkage or enlargement of the microchannels over time, microchannels were formed in ECM hydrogels, with and without carbon foam support, and the microchannel diameters were measured over time. The constructs with microchannels were maintained in 1 $\times$ DMEM (Cellgro) supplemented with 10% v/v fetal bovine serum (FBS; HyClone Laboratories), without perfusion. Five to fifteen microchannels were followed from 10 min to 161 h after polymerization and imaged using brightfield microscopy ($\times 100$ total magnification). Microchannel diameters were compared to original microchannel-forming rod diameters (i.e. 400 μm), using ImageJ software.

2.2. Measurement of material wettability

A polydimethylsiloxane (PDMS) flow chamber was fabricated to house the 3D construct (Fig. 1C). It was necessary to determine wettability between the ECM hydrogel and the PDMS, and between the ECM hydrogel and the carbon foam, to ensure proper sealing in the flow chamber. PDMS sheets were fabricated by mixing an elastomer base and curing agent (Sylgard) in a 10:1 ratio. After autoclave sterilization, the sheets were treated with either 63 wt% nitric acid or 25% v/v glutaraldehyde and rinsed in phosphate-buffered saline (PBS). Untreated sheets acted as the negative control. A 15 μl droplet of water or prepolymerized ECM hydrogel (as previously prepared) was deposited onto each PDMS sheet at room temperature. The camera (Keyence Digital Microscope VHX-600 or Panasonic Lumix DMC-ZS19) was placed in line with the PDMS sheets, where side-view images were captured at 45 s intervals. This process was repeated for each surface treatment in triplicate, for both water and ECM hydrogel. Contact angles (θ) were measured at the ECM hydrogel–

PDMS interface on both sides of the droplet, using the angle feature in ImageJ software. The process and measurements were repeated on a carbon sheet with the same composition as the carbon foam. Acute contact angles ($\theta \approx 90^\circ$) corresponded to wetting of the ECM hydrogel and PDMS, indicating that the hydrophilic surface was sufficient for proper sealing between the two materials. Obtuse contact angles ($\theta > 90^\circ$) indicated hydrophobicity of the PDMS.

Tensile testing of a 30 μL ECM hydrogel droplet was performed to confirm hydrogel adhesion to the PDMS. The droplet was sandwiched between two PDMS sheets attached to the actuator and platform of a dynamic mechanical analyser (RSA G2, TA Instruments) and allowed to polymerize. Tension was applied at a constant linear rate of 0.25 mm/s until separation of PDMS and ECM occurred. The test was replicated three times and a 10-element, moving average filter was used to find the separation force.

2.3. Design and construction of the flow-perfusion bioreactor

The flow-perfusion bioreactor was designed such that the ECM hydrogel could be polymerized inside the device without contamination or leaks. Transparency of the flow chamber was an additional device feature that enabled microscopic evaluation of samples in real time. The bioreactor device was composed of three pieces: an inlet flow port; an outlet flow port; and a bridge that housed the ECM hydrogels and was made of PDMS, i.e. the PDMS flow chamber; the latter were formed in the same manner as PDMS sheets, except fabrication occurred in a stainless steel mould ($10 \times 2.4 \text{ mm}^2$ cross-section). The bioreactor was connected to a medium reservoir and a peristaltic pump produced continuous forward-moving flow (Fig. 1C).

Volumetric flow rates (q) were measured and flow was assumed to be divided equally among the four microchannels, which were embedded in the 3D construct. The average microchannel radius (r) varied (125–200 μm), depending on the diameter of the microchannel-forming rods. The Reynold's number (Re), the ratio between inertial and viscous forces that indicates the type of flow (laminar or turbulent), was estimated from the volumetric flow rate through a single microchannel, according to the equation: $Re = 2qpr / \mu A$. The culture medium density (ρ) was volumetrically weighed to be approximately 995 kg/m^3 and the microchannel cross-sectional area (A) was derived from the microchannel-forming rod diameter, i.e. $A = (4.9 \times 10^{-4}) - (12.6 \times 10^{-4}) \text{ cm}^2$. The pressure gradient (p), an indicator of the construct's resistance to flow, was measured using the equation $p = P_{\text{downstream}} - P_{\text{upstream}} = \rho g (H_{\text{downstream}} - H_{\text{upstream}})$, where H is the height of medium in each Pitot tube and g is the standardized gravity value at 9.81 m/s^2 . The Pitot tubes were fabricated to measure the static pressure across the bioreactor.

2.4. Measurement of cell viability in the bioreactor system

Human breast cancer epithelial cells (MDA-MB-231) were cultured in DMEM supplemented with 10% v/v FBS and 600 $\mu\text{g/ml}$ G418 (HyClone Laboratories). Fibroblasts from normal breast (NAFs) were isolated after IRB approval, as previously described (Sadlonova *et al.*, 2005), and cultured in DMEM supplemented with 10% v/v FBS. MDA-MB-231 cells and NAFs were mixed into the ECM hydrogel solution at a 2:1 ratio. ECM

hydrogel polymerization followed, as described above. Three different cell–ECM preparations were fabricated: (a) solid, without microchannels (2×10^5 MDA-MB-231 cells + 1×10^5 NAFs/100 μ l ECM); (b) with microchannels embedded, but no flow (2×10^6 MDA-MB-231 cells + 1×10^6 NAFs/100 μ l ECM); and (c) with microchannels embedded and with convective flow (230.6 μ l/min volumetric flow rate, 2×10^6 MDA-MB-231 cells + 1×10^6 NAFs/100 μ l ECM). Solid cell–ECM preparations were placed in eight-well chamber slides, while microchannel-embedded cell–ECM constructs were prepared in the PDMS flow chamber with a carbon foam structural support, as described above. All cell–ECM preparations were maintained in low-serum medium (DMEM + 0.05% v/v FBS) for 1, 3 or 7 days. Solid cell–ECM preparations were maintained in 100 μ l medium/well, which was replaced with fresh medium every 3 days and the day after initial plating. Cell–ECM preparations with microchannels, but without perfusion, were maintained in the PDMS flow chamber and completely submerged in 20 ml medium in a 10 cm culture dish (one preparation/dish) for 7 days. Cell–ECM preparations with microchannels and perfusion were maintained in the PDMS flow chamber connected to the bioreactor and microphysiological pump and perfused by 30 ml medium, which was continuously circulated through a medium reservoir for 7 days. Cell–ECM preparations were subsequently fixed in neutral-buffered formalin, processed to paraffin and sectioned for haematoxylin and eosin (H&E) staining; three to five replicate cultures/constructs were completed for each condition and time point.

Autofluorescence of H&E-stained sections was used to determine overall viability of both cell types, as previously described (Hennings *et al.*, 2009). Briefly, photomicrographs of H&E-stained sections were acquired (SPOT Flex camera and SPOT basic software v. 5.0), using brightfield (25 ms exposure) and fluorescence microscopy (FITC filter set, 480 nm excitation, 4000 ms exposure; Zeiss Axiovert 35 with PlanF LWD $\times 40/0.60$ lens). Each image was corrected to a maximum brightness of 100 and background was subtracted, using a rolling ball status of 50 pixels, in ImageJ software. Following brightness correction and background subtraction, the intensity of auto-fluorescence of each cell was measured. All the cells in two complete histological cross-sections of each construct/culture were assessed, with an average of 516 (range 335–776) cells measured/construct or culture. Threshold intensity for determining live vs dead was derived by creating solid cell–ECM (MDA-MB-231 + NAF) preparations using dead cells only, a 1:1 ratio of dead and live cells, and live cells only. The cells were killed by treatment with 0.1 N HCL and heat at 60 °C for 2 h each. Receiver operating characteristics (ROC) analysis was used to identify a pixel intensity cut-off value for live vs dead that provided maximum possible sensitivity (90.3%) and specificity (86.96%) (Fig. 3A), as previously described (Hennings *et al.*, 2009). Using this pre-established cut-off (i.e. a pixel intensity of 16.35), cells in 3D cell–ECM preparations maintained for 1, 3, and 7 days were determined to be living or dead based on the autofluorescence intensity measured.

2.5. Statistical analyses

Groups of data representing three values at a minimum were denoted as average $\pm$ standard deviation (SD) or standard error of the mean, when appropriate. Using Student's t-test and assuming unequal variances, differences between two groups were significant at $p < 0.05$. ANOVA single-factor test with Tukey's multiple comparison test was used for more than

two data groups, with significance set at $p < 0.05$. Statistical analysis was accomplished using the data analysis package for Microsoft Excel or Graphpad Prism 6.

3. Results

The flow–perfusion bioreactor system included a construct consisting of a collagen–Matrigel mixture, supported by an inert carbon foam and featuring prefabricated microchannels for perfusion via a connected peristaltic pump. MDA-MB-231 cells and NAFs were embedded within the ECM hydrogel and their viability was monitored over time.

3.1. Characterization of the 3D construct components

Two important features of the 3D constructs were the incorporation of carbon foam and microchannels. Elemental mapping of the carbon foam surfaces using EDS exhibited carbon as the primary element, with minimal oxygen, present at 93.3 and 6.7 wt%, respectively. A backscatter electron microscopic image showed grey carbon struts with white oxygen deposits (Fig. 1A). Additionally, microchannels were formed successfully from 400 μm diameter stainless steel rods and their diameters over time were compared for ECM hydrogels, with and without a carbon foam support structure, as well as with and without a glutaraldehyde coating on the microchannel-forming rods. ECM hydrogels were maintained in DMEM without flow. At the end of 161 h, the only microchannels that remained patent were those formed from glutaraldehyde-soaked rods in carbon-supported ECM hydrogel (Fig. 2). Within 10 min of formation, the diameter of the microchannels formed from glutaraldehyde-coated rods was $331 \pm 82 \mu\text{m}$. There was no statistically significant difference in the diameters of these microchannels over the next 161 h.

3.2. Material wettability

Dynamic contact angle (θ), an indicator of ECM hydrogel wettability to PDMS flow chambers and carbon sheets, was measured for different surface treatments. In particular, the advancing contact angle (maximum angle, θ_{adv}) and the receding contact angle (minimum angle, θ_{rec}) describe the rearrangement of atoms inside the liquid and are attributed to surface roughness (Yuan and Lee, 2013). For PDMS sheets, nitric acid surface treatment significantly decreased the contact angle, resulting in a more hydrophilic surface (Table 1). For carbon sheets, glutaraldehyde surface treatment decreased the contact angle, indicating an increase in wettability and a more hydrophilic surface, whereas a nitric acid surface treatment did not significantly change the contact angle (Table 1).

A separation force was applied to a ECM hydrogel droplet sandwiched between two PDMS platens. The droplet consistently broke apart near its centre before the droplet detached from the PDMS sheets at $1.43 \pm 0.19 \text{ mN}$. This indicated that adhesive forces were greater than cohesive forces. A stress–strain relationship was not reported. Comparing the untreated PDMS sheets with surface-treated sheets resulted in no significant differences in separation force.

3.3. Laminar flow and pressure gradient measurements

From the volumetrically measured flow rates in a single microchannel, the Reynold's number was found to be significantly smaller than the critical Reynold's number (Table 2). Additionally, the pressure gradient was found to be 49 ± 2 Pa (0.37 ± 0.01 mmHg) for maximum fluid flow across the bioreactor.

3.4. Cell viability in co-cultures

Over time, viability of cells in solid cell–ECM preparations (no microchannels) showed a marked decrease, as indicated by an increase in cell autofluorescence. The average percentage of living cells in solid cell–ECM preparations decreased from a mean of 92.5% at day 1 of culture to 53.8% at day 3 and 44.8% at day 7 ($p < 0.0001$, ANOVA) (Fig. 3C). There was an increase in cell viability in cell–ECM constructs with four 400 μ m diameter microchannels, either with or without perfusion through the microchannels, compared to solid preparations ($p = 0.0009$, ANOVA) (Fig. 3D). This increase was even greater with perfusion at a volumetric flow rate of 240.6 μ l/min ($p = 0.001$, ANOVA), and the perfused constructs were found to have a significant increase in viability when compared to the constructs with microchannels without perfusion ($p = 0.05$, ANOVA). The average percentage of viable cells at day 7 in solid cell–ECM preparations was 44.8%, while that in constructs with microchannels without perfusion was 68.4%, and in constructs with perfusion through the microchannels via the bioreactor was 90.5%.

4. Discussion

Physiologically relevant *in vitro* tumour surrogates require ECM volumes that accommodate an appropriate 3D morphology of embedded cells. The volume of the hundreds to thousands of cells required for breast cancer surrogates may exceed the diffusion limit of oxygen and nutrients (100–200 μ m). Thus, through-channels and perfusion of the ECM hydrogel are necessary for cell viability and proliferation of larger cell volumes. Additional requirements include structural support of the viscous ECM hydrogel, system transparency for microscopic visualization and the ability to control flow of medium. Therefore, as a step in the development of a physiologically relevant, multicellular, breast cancer surrogate, the current study employed a thick (>500 μ m) 3D construct composed of breast carcinoma cells and breast fibroblasts embedded in an ECM hydrogel, a structural support and microchannels that were fabricated within a flow–perfusion bioreactor system. To our knowledge, the combination of supporting carbon foam and a collagen–Matrigel ECM hydrogel with multiple microchannels for perfusion is novel.

The ECM found in a breast carcinoma consists of a heterogeneous population of proteins that provide both structural support and functional cues to the surrounding cells. The composition of the ECM as well as the concentrations of ECM components are altered in breast cancer in comparison to the normal breast, due to the desmoplastic stromal response associated with most breast cancers (Lochter and Bissell, 1995). In addition, the ECM composition varies among different breast cancers, yet there are many commonalities. These include collagen type I, which is the major ECM component of most breast carcinomas (Egeblad *et al.*, 2010), and collagen type IV, laminin, entactin and heparin sulphate

proteoglycans, which make up the basement membrane (Coopman *et al.*, 1991). These components are all found to be increased in breast cancer stroma and may play a role in metastatic potential (Naba *et al.*, 2014). To provide a physiologically relevant ECM, our construct included a mixture of bovine collagen I and growth factor-reduced Matrigel, which contains many of the other ECM components increased in cancer, including those mentioned above (Kleinman and Martin, 2005).

The ECM hydrogels were supported by carbon foam to minimize hydrogel contraction and maintain microchannel patency. The large volume of ECM hydrogel necessitated an inert structural support, particularly since the ECM hydrogel would not support its own weight. This novel feature of our system was demonstrated to primarily contain carbon on its surface, suggesting an inert surface that would not induce cell toxicity. Breast cancer epithelial cell and breast fibroblast viability was maintained in 3D constructs with the carbon foam, supporting the inert nature of the carbon. Other researchers have corroborated this conclusion when they showed little to no cell toxicity *in vitro* and no foreign body reaction to carbon *in vivo* (Pec *et al.*, 2010).

ECM hydrogel perfusion required that flow be confined to the microchannels, with a watertight seal between the ECM hydrogel and support structures (carbon foam and PDMS flow chambers) for monitoring of accurate flow rates. Flow rates resulted in a low Reynold's number, which predicts laminar flow through each microchannel. Contact angle and separation force measurements confirmed that surface treatment of carbon and PDMS resulted in hydrophilicity, which indicated wetting and adherence of the ECM hydrogel. While the mean hydrogel–PDMS contact angle of $80 \pm 9^\circ$ was less than the cut-off for hydrophilicity ($\theta = 90^\circ$), the advancing contact angle of $96 \pm 5^\circ$ was above the cut-off, suggesting that a stronger surface treatment may be necessary to ensure a tight seal between the PDMS and ECM hydrogel. Separation force measurements confirmed adhesion-dominated cohesion forces, thus indirectly indicating a seal between the PDMS and ECM hydrogel.

Perfusion required open, parallel microchannels to enable nutrient diffusion throughout the entire 3D volume. Experimental analysis showed that microchannels maintained patency during static conditions, with no statistically significant change in diameter for up to 161 h when the microchannels were supported by carbon foam and formed by glutaraldehyde-coated rods. Unsupported microchannels (i.e. no carbon foam) and supported microchannels without a glutaraldehyde coating on the microchannel-forming rods showed a rapid decrease in diameter and complete closure between 38 and 161 h. The relatively quick closure of the microchannels when glutaraldehyde was not employed may be due to the lack of collagen crosslinking along the microchannel wall. These results suggest that a combination of carbon foam support and glutaraldehyde coating on the microchannel-forming rods is necessary to slow ECM hydrogel contraction and maintain microchannel patency.

After characterization of the non-cellular materials in the 3D constructs, breast cancer cells and breast fibroblasts were incorporated, to determine whether viability could be maintained in the flow–perfusion bioreactor system. It was found that when 3D constructs were perforated with microchannels and perfused with medium, overall cell viability was

increased when compared to solid cell–ECM preparations and cell–ECM preparations without perfusion. Interpretation of these data is somewhat limited by differences in the volume of medium used for solid 3D cell–ECM preparations vs those with microchannels. Because of the use of eight-well chamber slides to support the solid cell–ECM preparations, the ratio of medium volume to the number of cells in the cultures was higher in solid preparations, with a ratio of 3×10^6 cells/ml medium, than in preparations with microchannels, with a ratio of 1.5×10^5 cells/ml medium in preparations without perfusion and a ratio of 1×10^5 cells/ml medium in preparations with perfusion. It is possible that this difference may affect the availability of nutrients and could contribute to lower viability in solid preparations. This is somewhat mitigated by the fact that, in the solid 3D constructs, the medium was changed every 3 days to replenish nutrients throughout culture. In addition, there was no major change in pH of the medium, as indicated by the absence of a visible colour change in the phenol red indicator. A drop in pH is a major factor that signifies the need for medium replacement (Freshney, 2011). Although the higher cell number:medium volume ratio may have contributed to the decreased viability in the solid preparations, we believe that it is more likely to be due to poor diffusion of nutrients into the solid preparations, which has been identified as a limitation of 3D culture (Kim *et al.*, 1998). The diffusion distance of oxygen is $<100\text{--}200\text{ }\mu\text{m}$ (Gibot *et al.*, 2010; Rouwkema *et al.*, 2008) and other larger nutrients would likely diffuse to a lesser extent. It is unlikely that passive diffusion alone would be sufficient to support viability by effectively delivering nutrients and removing waste in the solid cultures. The increase in viability seen with flow vs without flow in cell–ECM preparations with microchannels can be more confidently attributed to the addition of continuous medium perfusion, given that the cell number:medium volume ratio is similar in the two conditions. Therefore, it is likely that the presence of microchannels alone increased cell viability in the 3D cultures, and viability was further enhanced by the presence of continuous perfusion of medium.

The flow–perfusion bioreactor and 3D construct system that has been described here incorporates various features, some of which are unique, that will facilitate the tissue engineering of more physiologically relevant *in vitro* 3D breast cancer surrogates. The 3D construct has an enhanced volume compared to lab-on-a-chip systems, which will enable the engineering of larger tumour volumes, with different cell types arranged in an appropriate architecture, providing cross-talk between multiple cell types. Incorporation of the ECM hydrogel further increases the physiological relevance of the system compared to traditional 2D cell culture. The presence of the microchannels allows for perfusion of the construct and enhanced cell viability, and also serves as a platform for the engineering of a microvasculature complete with endothelial cells and 'neovascularization', which is vital to cancer development and progression (Weis and Cheresh, 2011).

5. Conclusions

We have fabricated a novel flow–perfusion bioreactor with 3D construct system that provides a platform for tissue engineering of physiologically relevant and architecturally correct *in vitro* breast cancer surrogates. A unique feature of our system is the combination of supporting carbon foam and a collagen–Matrigel ECM hydrogel, with multiple microchannels for perfusion to allow for long-term cell viability *in vitro*. We showed that the

addition of inert carbon foam enabled the fabrication of thicker 3D constructs and microchannels that maintained their integrity over time. We further demonstrated that the ECM hydrogel was adherent to the carbon foam and the PDMS flow chamber for a tight seal. When human breast carcinoma and stromal cells were embedded in the construct, it was shown that the microchannels with convective flow provided enhanced cell viability.

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Figure 1.

The cancer construct is supported and maintained by carbon foam, microchannels and a perfusion–flow bioreactor. (A) Backscatter scanning electron microscopy image of the carbon foam shows grey carbon struts with white oxygen deposits; scale bar = 500 μm . (B) Close-up of microchannel-forming rods penetrating through the 3D construct composed of a carbon foam and ECM hydrogel; scale bar = 500 μm . (C) Perfusion–flow bioreactor with 3D construct is housed inside a PDMS flow chamber (transparent sleeve); arrows, flow direction of working fluid. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 2.

Effect of carbon foam structural support and rod coating on microchannel diameter. Comparison of microchannel diameter in ECM hydrogel alone (hydrogel), carbon-support ECM hydrogel without glutaraldehyde-coated rods (CF NG) and carbon-support ECM hydrogel with glutaraldehyde-coated rods (CF GS) over 161 h after microchannel formation. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 3.

Cell viability is increased in the constructs in the bioreactor system compared to solid preparations. (A) Autofluorescence intensity cut-off determination, using dead cells only, a 1:1 mixture of dead and live cells, and live cells only: black bars, mean and SD of the autofluorescence intensity for each condition; red vertical line delineates the cut-off with maximum sensitivity and specificity, used to determine viability. (B) Representative autofluorescence images of H&E-stained histological sections from a solid cell-ECM preparation and a microchannel-embedded construct with convective flow after 7 days: images obtained by fluorescence microscopy (left) and by brightfield microscopy (right) of the same microscopic field for each of the two culture conditions are shown; red arrows indicate living cells and white arrows indicate dead cells, as determined by intensity of autofluorescence. (C, D) Cell viability, as determined by autofluorescence intensity of solid preparations (C) after 1, 3 and 7 days of culture and (D) comparing solid preparations and microchannel-embedded constructs, with and without flow, after 7 days: $n = 3$ replicate solid cultures at 7 days; $n = 4$ replicate solid cultures at 1 day; replicate constructs with

microchannels but without flow; and replicate constructs with microchannels and flow; $n = 5$ replicate solid cultures at 3 days; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; ANOVA with Tukey's multiple comparison test. [Colour figure can be viewed at wileyonlinelibrary.com]

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Table 1

Effect of surface treatment on advancing (θ_{adv}), mean (θ) and receding (θ_{rec}) contact angles, reported as mean $\pm$ SD ($n = 3+$)

	No coating ($^{\circ}$)			50 v/v% Glutaraldehyde ($^{\circ}$)			63 wt% Nitric acid ($^{\circ}$)		
	θ_{adv}	θ	θ_{rec}	θ_{adv}	θ	θ_{rec}	θ_{adv}	θ	θ_{rec}
PDMS	116 $\pm$ 8	109 $\pm$ 7	102 $\pm$ 2	107 $\pm$ 0	103 $\pm$ 3	100 $\pm$ 1	96 $\pm$ 5	80 $\pm$ 9	74 $\pm$ 7
Carbon	125 $\pm$ 7	116 $\pm$ 8	109 $\pm$ 9	45 $\pm$ 8	28 $\pm$ 7	23 $\pm$ 3	122 $\pm$ 2	116 $\pm$ 5	107 $\pm$ 3

Table 2

For a single microchannel of two different sizes that perforated the 3D constructs, minimum and maximum volumetric flow rates and Reynold's number are reported

Diameter, d (μm)	Flow rate, q ($\mu\text{l}/\text{min}$)	Reynold's number, Re
250	11.1	0.26
	307.2	7.22
400	11.1	0.16
	947.6	13.89