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A gel-free 3D microfluidic cell culture system

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ABSTRACT

3D microfluidic cell culture systems offer a biologically relevant model to conduct micro-scale mammalian cell-based research and applications. Various natural and synthetic hydrogels have been successfully incorporated into microfluidic systems to support mammalian cells in 3D. However, embedment of cells in hydrogels introduces operational complexity, potentially hinders mass transfer, and is not suitable for establishing cell-dense, ECM-poor constructs. We present here a gel-free method for seeding and culturing mammalian cells three-dimensionally in a microfluidic channel. A combination of transient inter-cellular polymeric linker and micro-fabricated pillar arrays was used for the *in situ* formation and immobilization of 3D multi-cellular aggregates in a microfluidic channel. 3D cellular constructs formed this way are relieved of hydrogel embedment for cellular support. Two mammalian cell lines (A549 and C3A) and a primary mammalian cell (bone marrow mesenchymal stem cells) were cultured in the gel-free 3D microfluidic cell culture system. The cells displayed 3D cellular morphology, cellular functions and differentiation capability, affirming the versatility of the system as a 3D cell perfusion culture platform for anchorage-dependent mammalian cells.

1. Introduction

Microfluidic cell culture systems offer many advantages for applications in cell-based research. The precision of microfabrication allows the presentation of a controllable and reproducible microenvironment for mammalian cell culture formerly unattainable by standard tissue culture techniques [1]. The ease of multiplexing for high throughput analyses [2] and possibilities of integrating microfluidic components, such as concentration gradient generators [3,4] and computer-controlled on-chip valves, pumps and analytical systems [1,5], further add to the appeal of microfluidic cell culture systems. Transparent microfluidic platforms are also compatible with optical-based *in situ* assays that utilize fluorescence reporters and high resolution imaging modalities, such as confocal microscopy, to probe cellular events or monitor cellular responses [6]. Hence, microfluidic cell culture systems have found applications in many areas of mammalian cell-based research, such as cell migration studies [7], gene expression analysis [8], cellular function and differentiation studies [9], drug metabolism and toxicity testing [10], and disease diagnosis [11].

To improve the biological relevance of microfluidic cell culture systems, there is emphasis to move from two-dimensional (2D) cell culture (where cells are cultured as a monolayer on a flat substrate) to three-dimensional (3D) cell culture (where cells are supported in all directions by either neighboring cells or extracellular matrix (ECM)) in microfluidic systems [12,13]. Cells cultured in 3D display gene expression profiles and biological activities that resemble the *in vivo* situation more closely than the cells cultured in 2D monolayer [14]. A common strategy to effect 3D cell culture is to embed cells three-dimensionally in hydrogels [13,15]. Here, we refer to hydrogel as a network of hydrophilic polymer chains that are water-insoluble [16]. Cells are first suspended in liquid hydrogels, and then embedded three-dimensionally when gelation sets in. Various natural and synthetic hydrogels have been incorporated into microfluidic cell culture systems to support cells in 3D [13,17,18]. However, there are several limitations associated with the use of

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hydrogels to effect 3D cell culture in microfluidic systems. Animal-derived hydrogels (*e.g.*, collagen) can be variable in composition and properties [14,19], while some synthetic hydrogels (*e.g.*, poly(ethylene glycol) (PEG)) require cytotoxic ultra-violet photo-polymerization [15,20]. Mass transport of oxygen and nutrients through dense hydrogels, which are required to mechanically withstand fluid perfusion, may also be inefficient [21]. Furthermore, hydrogels need to be spatially localized within the microfluidic channel so as not to impede fluid flow. This requirement may impose additional operational steps, such as sealing of the microfluidic channel after hydrogel incorporation [22] or using hydrodynamic focusing for *in situ* gelation within the microfluidic channel [17,23], which can exacerbate the design and operational complexity of the microfluidic system. More importantly, the use of hydrogels to embed cells may not be ideal for forming 3D *in vitro* models that are cell-dense and ECM-poor. For instance, solid tumor models are predominantly established by cell aggregation into spheroids rather than hydrogel embedment [24,25]. Liver models based on hepatocyte spheroids also exhibited better polarity and differentiated functions as compared to models that are based on hydrogel embedment [26,27]. Thus, there is a need to develop a gel-free microfluidic system to establish these cell-dense and ECM-poor 3D *in vitro* models in microfluidics.

We present here a novel method for seeding and culturing mammalian cells three-dimensionally in microfluidic systems without the use of hydrogels. A combination of transient inter-cellular polymeric linker (hereafter referred to as "inter-cellular linker") [28] and micro-fabricated pillar arrays [13] was used for the *in situ* formation and immobilization of 3D multi-cellular aggregates in a microfluidic channel. Operation of this gel-free 3D microfluidic cell culture system (hereafter referred to as "gel-free 3D-μFCCS") does not encounter the limitations nor requires additional operational steps that the above-described hydrogel-based systems do. In addition, 3D cellular constructs formed this way are relieved of hydrogel embedment for cellular support, giving a cell-dense construct. We demonstrated the biological versatility of this system by culturing two mammalian cell lines (A549 and C3A) and a primary mammalian cell (bone marrow mesenchymal stem cells (BMSCs)) in the gel-free 3D-μFCCS. All the cells were viable and displayed 3D cellular morphology. Evaluation of the cellular functions of C3A cells and differentiation capability of BMSCs demonstrated the utility of the system as a 3D cell culture platform for anchorage-dependent mammalian cells.

2. Materials and methods

2.1. Device fabrication

Microfluidic channels with micropillar arrays were designed using AutoCAD (Autodesk, USA) (Fig. 1a). The dimensions of the microfluidic channel were 1 cm (length) × 600 μm (width) × 100 μm (height); and each microfluidic channel has two inlets and one outlet. An array of 30 × 50 μm elliptical micropillars with a 20 μm gap size is situated in the center of the microfluidic channel, bounding a cell residence compartment that is 200 μm wide (Fig. 1c). Silicon templates were fabricated by standard deep reactive ion etching (DRIE) process (Alcatel, France). The microfluidic channels were then obtained by replica molding poly(-dimethylsiloxane) (PDMS) (Dow Corning, USA) on the silicon templates. The PDMS structures were plasma-oxidized in oxygen plasma for 1 min (125 W, 13.5 MHz, 50 sccm, and 40 millitorr) for irreversible bonding to glass coverslips before connecting to fluidic components (Upchurch, USA). One inlet of the microfluidic channel was connected to a cell reservoir, which comprised of a two-way valve with a luer connection (Cole–Palmer, USA) coupled to a 22G stainless steel hypodermic needle (Becton–Dickinson, USA) (Fig. 1b). The other inlet and outlet are for cell culture medium perfusion (Fig. 1b). The entire set-up was sterilized by autoclaving at 105 °C for 30 min.

2.2. Inter-cellular polymeric linker preparation

The inter-cellular polymeric linker, polyethyleneimine-hydrazide (PEI-hy), and its fluorescent version were synthesized and purified as described previously [28]. A concentration of 6 μM of PEI-hy in culture medium was used in this study.

2.3. Cell seeding in gel-free 3D microfluidic cell culture system (3D-μFCCS)

Cells were first modified with 0.5 mM of sodium periodate ($NaIO_4$) (Sigma) in test tubes at 4 °C in the dark [28]. Cell seeding was performed by withdrawing a suspension (50 μl) of modified cells (6 million ml^{-1}) and dissolved inter-cellular linker (6 μM PEI-hy) from the cell reservoir, via the outlet using a withdrawal syringe pump at a flow rate of 0.03 ml h^{-1}. Upon filling up of central cell compartment, the cell reservoir was closed and culture medium infused from the inlet at a flow rate of 0.03 ml h^{-1}.

2.4. Cell culture

All cell culture components are purchased from GIBCO, Invitrogen, USA unless otherwise stated. A549 cells (ATCC, USA) were cultured in F12K supplemented with

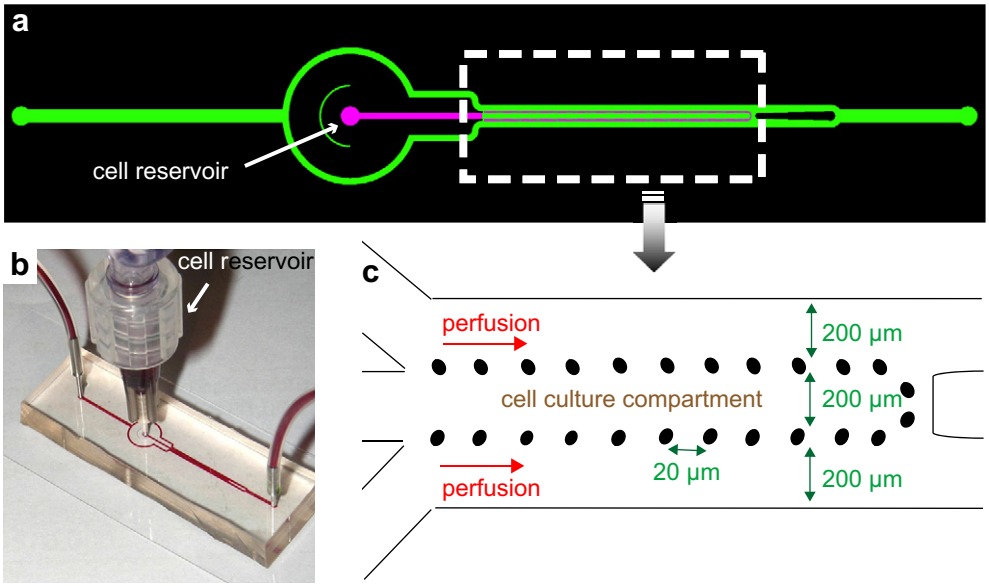

Fig. 1. Design of the gel-free 3D microfluidic cell culture system (3D-μFCCS). (a) The gel-free 3D-μFCCS has two inlets (one for culture medium infusion, one as cell reservoir) and one outlet. (b) Prototype of the gel-free 3D-μFCCS. (c) Dimensions of the 3D-μFCCS. Micropillars divided the microfluidic channel into a central cell culture compartment and two-side channels for perfusion of culture medium.

10% fetal calf serum, 1.5 g L^{-1} sodium bicarbonate, 2 mM L-glutamine, 100 units ml^{-1} penicillin and 100 g ml^{-1} streptomycin. C3A cells (ATCC, USA) were cultured in Minimum Essential Medium supplemented with 10% fetal calf serum, 1.5 g L^{-1} sodium bicarbonate, 1 mM sodium pyruvate, 100 units ml^{-1} penicillin and 100 g ml^{-1} streptomycin. Bone marrow stem cells were harvested from the bone marrow of male Wistar rats and cultured in low glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and 1.5 g L^{-1} sodium bicarbonate. Osteogenic medium was prepared by supplementing basal medium with 100 nM dexamethasone, 50 μM ascorbic acid 2-phosphate and 10 mM β-glycerophosphate (Merck, Singapore).

2.5. Perfusion culture in gel-free 3D-μFCCS

The cells are cultured in a one-pass perfusion manner with a syringe pump (Cole–Palmer) at 0.03 ml h^{-1}. The microfluidic system was placed onto a heating plate (MEDAX GmbH & Co. KG, Germany) maintained at 37 °C throughout the culture period in a sterile hood. Sixty millimolar of Hepes buffer (GIBCO, Invitrogen, USA) was added to the culture medium to maintain its pH at 7.4–7.6.

2.6. Cell viability staining

Cell viability of A549, C3A, and BMSCs after 3 days of perfusion culture in the gel-free 3D-μFCCS was assessed by perfusing 5 μM of Calcein AM (Molecular Probes, USA) and 25 μg ml^{-1} of propidium iodide at 0.5 ml h^{-1} for 30 min and viewing immediately by confocal microscopy (Fluoview 300, Olympus, Japan).

2.7. F-actin staining

F-actin distribution in all cell types was assessed after 3 days of perfusion culture in the gel-free 3D-μFCCS. *In situ* F-actin staining was performed after fixation with 3.7% paraformaldehyde (PFA) (30 min) by infusing the microfluidic channel with 0.5% Triton-X 100 (USB Corp, USA) (30 min), 0.2% bovine serum albumin (BSA) (30 min), 0.2 μg ml^{-1} of TRITC-phalloidin (Invitrogen, Singapore) (20 min) and 1× PBS (15 min) at 0.5 ml h^{-1}. 2D monolayer cultures were fixed with 3.7% PFA (15 min) and stained by incubating with 0.5% Triton-X 100 (10 min), 0.2% BSA (15 min), and 0.2 μg ml^{-1} of TRITC-phalloidin (20 min).

2.8. Scanning electron microscopy (SEM)

SEM samples were prepared by bonding the PDMS microfluidic channels onto a polyethylene (PE) film (Diversified Biotech, USA) instead of a glass coverslip. The samples were fixed with 3.7% PFA before the PE film was peeled off to expose the microfluidic channel for SEM processing. Samples were sequentially dehydrated with ethanol series (25, 50, 75, 95 and 100%), and then platinum-sputtered (20 mA, 60 s) before viewing with a field-emission scanning electron microscope (JEOL, Japan).

2.9. Functional assessment of C3A

All functional data were normalized to cell number (DNA content) quantified using PicoGreen assay (Molecular Probes, USA) according to the manufacturer's protocol. Albumin production was quantified with a human albumin ELISA quantification kit (Bethyl Laboratories Inc, USA) in culture medium collected. For comparison with 2D culture, the culture medium of a confluent 2D monolayer incubated in static wells was collected for albumin quantification. Albumin production is expressed as total albumin (μg) collected in culture medium per million cells over 24 h. UDP-glucuronyltransferase (UGT) activity of C3A cultured in the gel-free 3D-μFCCS was determined by infusing 100 μM of 4-methylumbelliferone (4-MU) (Sigma, Singapore) for 6 h at 0.05 ml h^{-1}. The perfusate (300 μl) was collected and the metabolic product, 4-methylumbelliferyl glucuronide (4-MUG), was analyzed using capillary electrophoresis with laser induced fluorescence (CE-LIF) [29] detection (Prince Technologies B.V., Netherlands) at an excitation wavelength of 325 nm. UGT activity in confluent 2D monolayer cultures was determined by incubating the samples with 300 μl of 100 μM of 4-MU for 6 h before CE-LIF analysis. 4-MUG production is expressed as total 4-MUG (pg) collected in culture medium per million cells over 6 h. Data are represented as mean ± SD of two chips (3D-μFCCS) or two culture wells (2D monolayer).

2.10. von Kossa staining

BMSCs after 1 week of osteogenic induction were stained for calcium salt deposits by von Kossa staining. von Kossa samples were prepared by bonding the PDMS microfluidic channels onto a PE film instead of a glass coverslip, fixed with 3.7% PFA before the PE film was peeled off to expose the microfluidic channel. Effective washing could be achieved to avoid false positive staining. von Kossa staining was carried out with static incubation of the following: 5% silver nitrate solution (45 min), distilled water (DIW) (3 × 5 min), 5% sodium bicarbonate in 3.7% formaldehyde solution (8 min), DIW (3 × 5 min), 5% sodium thiosulfate (5 min) and DIW (3 × 5 min). Likewise, von Kossa staining was performed in 2D monolayer cultures after 1 week of osteogenic induction.

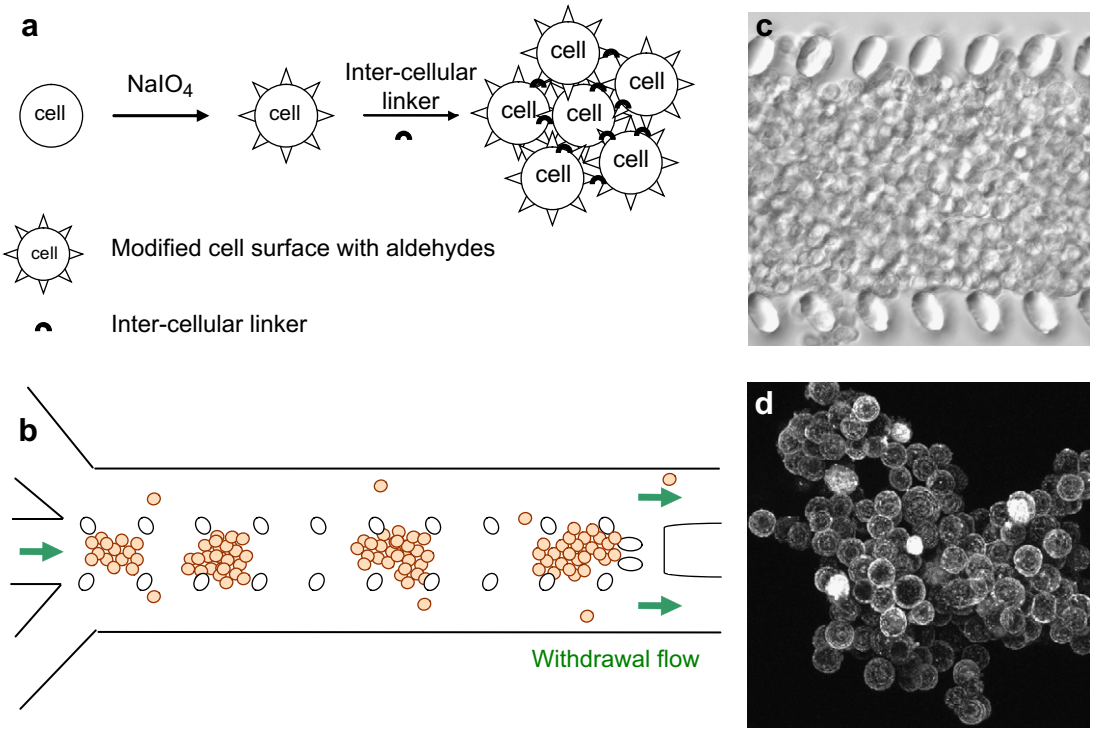

Fig. 2. Cell seeding into the gel-free 3D-μFCCS. (a) Schematic representation of the cell aggregation process. Cell surfaces modified by sodium periodate ($NaIO_4$) display aldehyde groups which react with the hydrazides on the inter-cellular linker to form multi-cellular aggregates. (b) Schematic representation of the *in situ* formation and immobilization of 3D multi-cellular aggregates in the microchannel. Cells were suspended in cell culture medium with dissolved inter-cellular linker and seeded into the microfluidic channel with a withdrawal flow at the outlet. (c) Transmission image of the cellular construct in the gel-free 3D-μFCCS after seeding. (d) Confocal image of cells aggregated with fluorescent inter-cellular linker, showing cells in the construct supported in 3D by neighboring cells.

Table 1
Optimized operation parameters for seeding cells into the gel-free 3D-μFCCS

Parameter	Optimal operation window
Cell density (million ml^{-1})	5–6
Linker concentration (μM)	6–8
Withdrawal flow rate (ml h^{-1})	0.03

3. Results

3.1. The gel-free 3D microfluidic cell culture system (3D-μFCCS)

The gel-free 3D-μFCCS was developed to effect *in situ* formation and immobilization of 3D multi-cellular aggregates in a microfluidic channel without the use of hydrogels. It consists of a microfluidic channel 1 cm (length) × 600 μm (width) × 100 μm (height) (Fig. 1a and b). An array of elliptical micropillars, 30 × 50 μm, spaced 20 μm apart, is situated in the center of the microfluidic channel. The geometrical design of the micropillar array was previously optimized to reduce clogging during cell seeding [13]. The micropillar array divides the microfluidic channel into a central compartment for cell culture and two side channels for perfusion of culture medium, each 200 μm wide (Fig. 1c). The central cell culture compartment is connected to a cell reservoir inlet for cell seeding, and the two side channels are connected to a perfusion system (Figs. 1b and 3a).

Before seeding, cells were first treated with sodium periodate to modify the sialic acids on cell-surface glycoproteins to generate aldehyde groups [28]. Modified cells were suspended in inter-cellular linker dissolved in cell culture medium and immediately introduced into the cell reservoir and seeded into the center compartment of the microfluidic channel with a withdrawal flow of 0.03 ml h^{-1} at the outlet. The inter-cellular linker consists of a polymeric molecule (polyethyleneimine) with multiple hydrazide arms [28]. During the seeding process, the aldehydes on modified cell surfaces reacted with the hydrazides on the inter-cellular linker to form a hydrazone covalent linkage, establishing cell–cell contacts resulting in cell aggregation (Fig. 2a and b). As the aggregates are withdrawn down the length of the microfluidic channel, they snowballed to form larger cell aggregates, which were then confined by the micropillar arrays and accumulated, forming a 3D cellular construct (Fig. 2c). When a fluorescent label was used to visualize the linkers, fluorescent rings could be seen on cell surfaces, affirming that the cells were aggregated by the linker and supported three-dimensionally by neighboring cells (Fig. 2d).

To effectively seed the 3D cellular aggregates into the microfluidic channel, optimization of various operational parameters was performed (Table 1) to ensure that the *in situ* formed cellular aggregates were large enough to be confined by the micropillar arrays and, yet, small enough to prevent clogging of the microfluidic channel. The size of the cellular aggregates can be modulated by the cell density and the inter-cellular linker concentration. A cell density of 5–6 million cells ml^{-1} at inter-cellular linker concentration of 6–8 μM was optimal for effective seeding. At higher cell densities or linker concentrations, massive clogging occurred at the inlet; while at lower cell densities and linker concentrations, ineffective aggregation of cells saw poor cell entrapment. For all subsequent studies, 6 million cells ml^{-1} at inter-cellular linker concentration of 6 μM was used. The withdrawal flow rate during cell seeding was optimized to be 0.02–0.05 ml h^{-1} to ensure high cell viability after the seeding process [13].

3.2. Perfusion culture of mammalian cells in the gel-free 3D microfluidic cell culture system (3D-μFCCS)

To investigate the biological versatility and potential of the gel-free 3D-μFCCS as a mammalian cell perfusion culture system, we

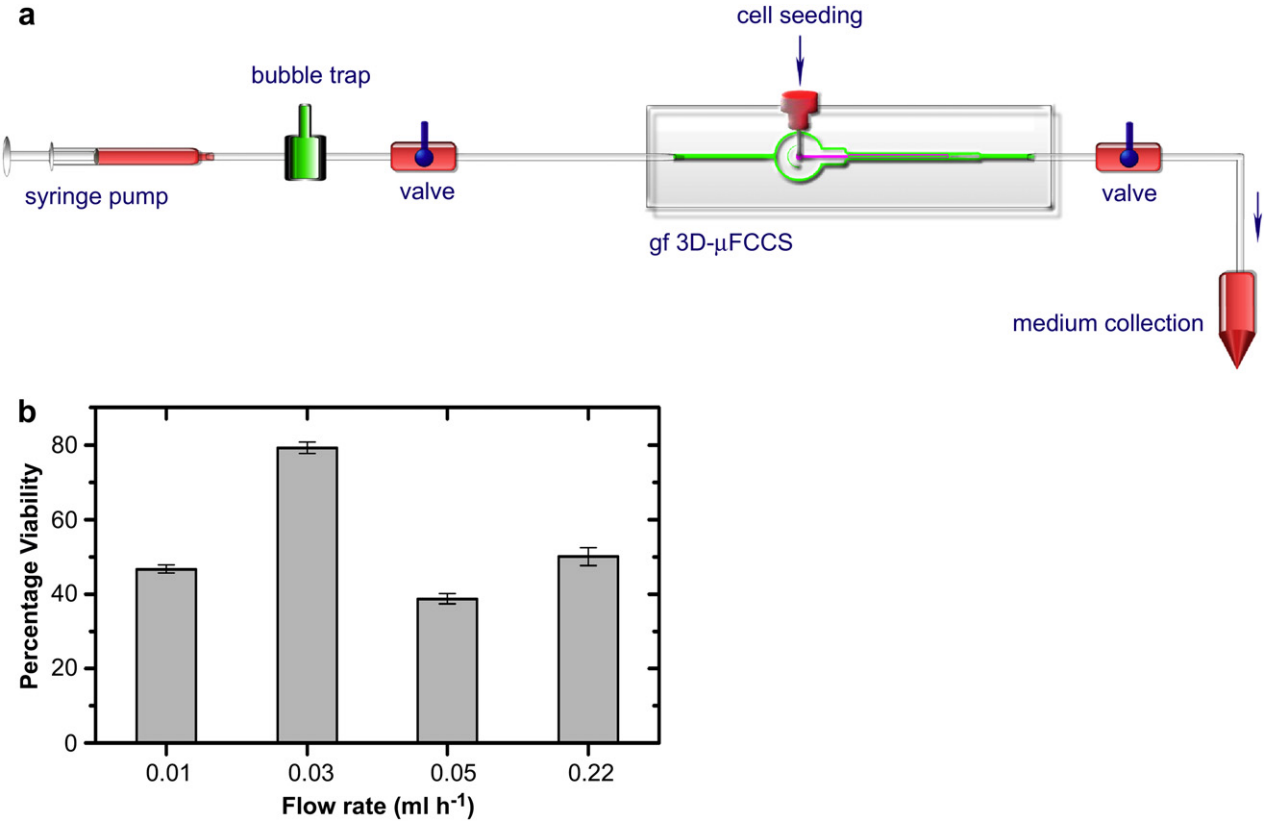

Fig. 3. Perfusion culture of the cells in the gel-free 3D-μFCCS. (a) Schematic representation of a one-pass perfusion culture system. (b) Optimization of perfusion culture flow rate for maximum cell viability.

cultured a few model anchorage-dependent mammalian cells, such as the human lung epithelial cell line A549, human liver cell line C3A, and rat primary progenitor cells (BMSCs), using a one-pass perfusion system (Fig. 3a). A one-pass perfusion system allows the maintenance of a constant cell culture microenvironment over time, without accumulation of metabolites or depletion of oxygen and nutrients that is experienced by re-circulating perfusion cultures [30]. The culture medium was oxygenated by passing through oxygen-permeable tubing before entering the bubble trap. The perfusion flow rate to culture the 3D cellular construct was 0.03 ml h^{-1}, optimized for maximal cell viability (Fig. 3b). Culture medium at the end of the perfusion circuit can be collected for assessment of cellular functions.

Cell viability after 3 days of perfusion culture was assessed with fluorescence viability staining and imaged by confocal microscopy. All three cell types, the human lung epithelial cell line A549, human liver cell line C3A, and rat primary progenitor cells (BMSCs) exhibited good cell viability (Fig. 4a, d and g). Actin visualization revealed a cortical actin distribution typical of the 3D cell morphology for A549 (Fig. 4b) and C3A cells (Fig. 4e), in contrast to the stress fibers seen in the 2D-cultured cells (insets). Actin labeling of the BMSCs showed less stress fibers within the large aggregates (Fig. 4h) than those present in the 2D cultures (inset). Closer examination of the A549 and C3A cells using SEM showed rounded cell morphology with gradual merging of the cell–cell boundaries indicative of increasing cell–cell interactions over time (Fig. 4c and f), while BMSCs remodeled into large and tight 3D aggregates with smooth surfaces (Fig. 4i). These results illustrated that all three cell types remained viable and maintained their 3D morphology during the 3-day perfusion culture in the gel-free 3D-μFCCS.

3.3. Functional maintenance of mammalian cells in the gel-free 3D microfluidic cell culture system (3D-μFCCS)

To further evaluate the gel-free 3D-μFCCS for mammalian cell culture, C3A cells were assessed for proliferation and cellular functions over 5 days of perfusion culture. Total DNA quantification showed that cell proliferation occurred in the gel-free 3D-μFCCS during culture (Fig. 5a). Synthetic and metabolic functions of C3A cells were quantified by measuring the albumin production and UDP-glucuronyltransferase (UGT) activity via 4-methylumbelliferyl glucuronide (4-MUG) formation, respectively. Albumin production by C3A cells in the gel-free 3D-μFCCS ranged from 4.0 to 5.2 μg albumin per million cells per day over the culture period (Fig. 5b), which was more than twice the amount (1.8 μg) produced by cells grown in a confluent 2D monolayer (dotted line, Fig. 5b). C3A cells

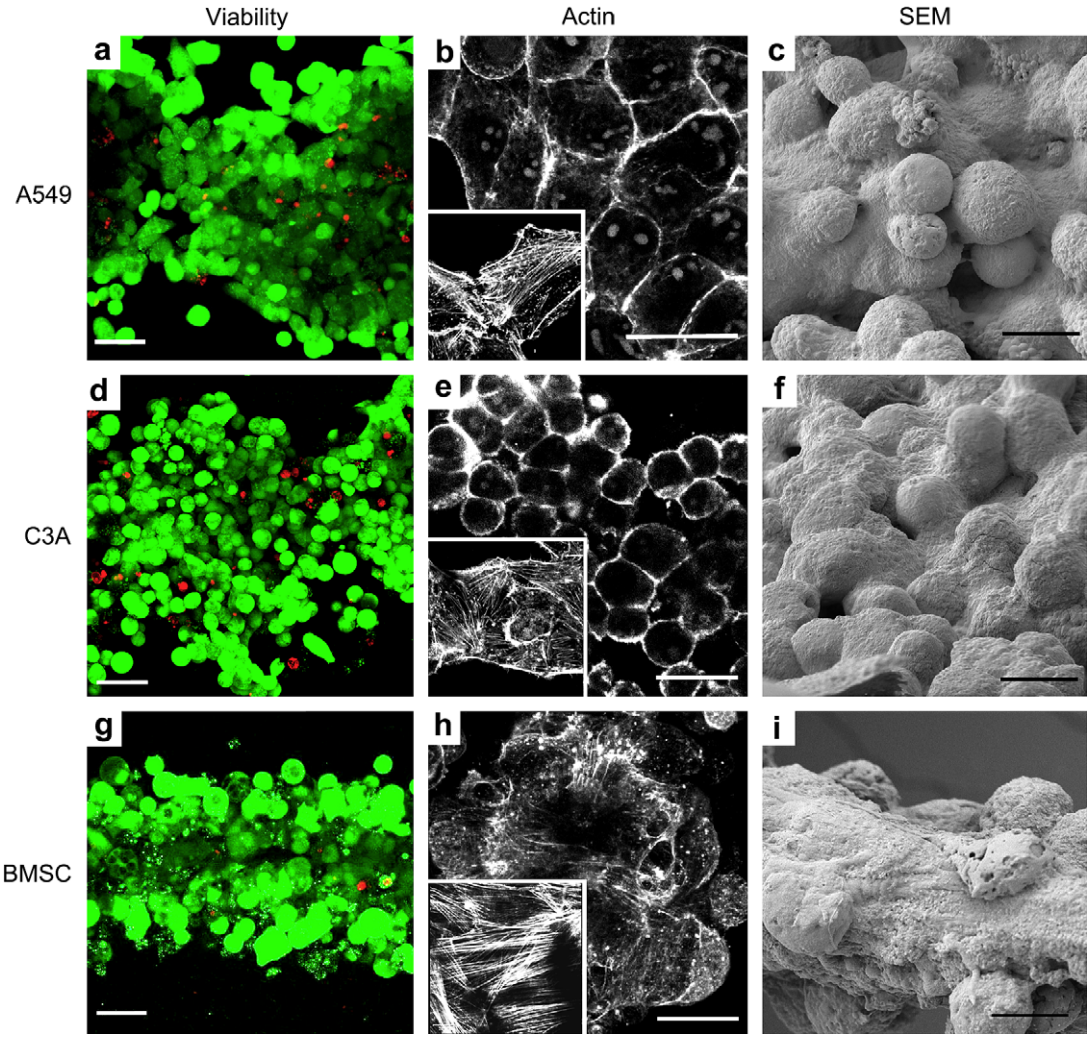

Fig. 4. Three cell types in the gel-free 3D-μFCCS are viable and maintained 3D morphology after 3 days of perfusion culture. (a), (d), and (g) Confocal images of cells stained with Calcein AM (which stains live cells green) and propidium iodide (which stains dead cells red) indicated good cell viability. Scale bar: 50 μm. (b), (e), and (h) Confocal images of F-actin staining showed cells with cortical distribution of actin, reminiscent of the 3D cell morphology. In contrast, cells in 2D show extensive stress fiber formation (insets). Scale bar: 20 μm. (c), (f), and (i) SEM images of cells illustrated rounded 3D cell morphology with gradual merging of cell–cell boundaries. Scale bar: 10 μm.

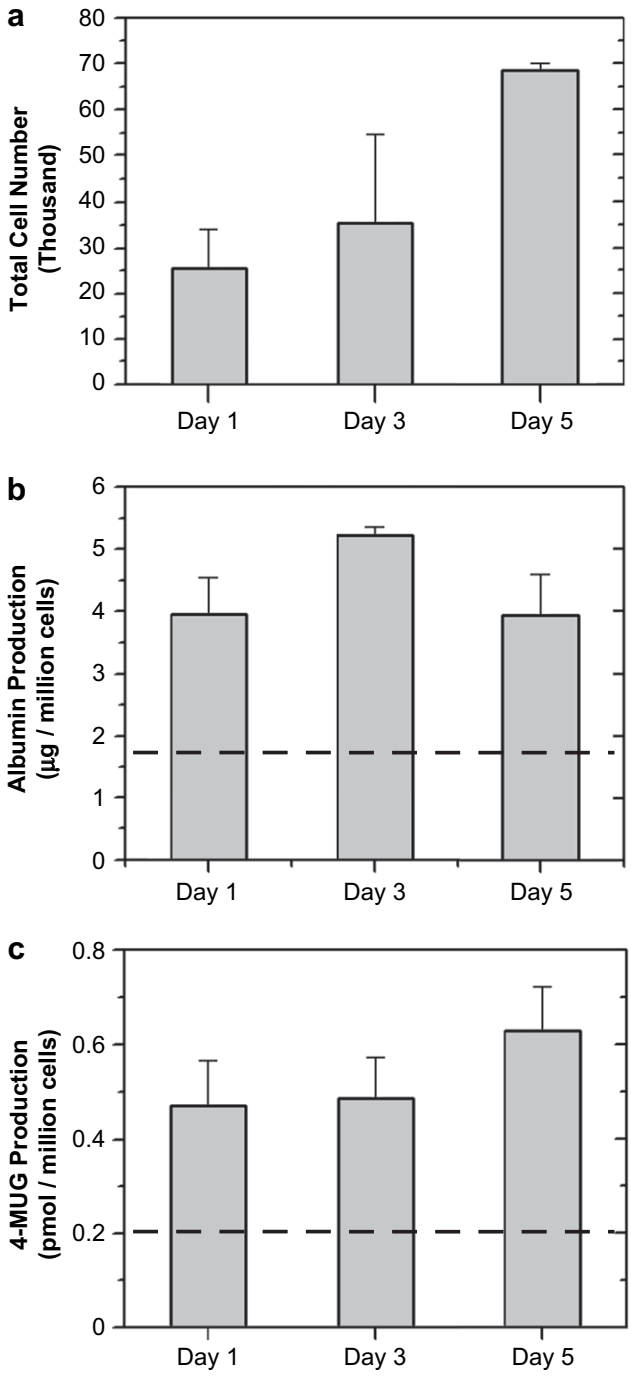

Fig. 5. C3A cells cultured in the gel-free 3D-μFCCS proliferated and exhibited good cellular functions. (a) Total cell number measured showed cell proliferation in the gel-free 3D-μFCCS. (b) and (c) Functional assessment of C3A cells cultured in the gel-free 3D-μFCCS by albumin production (b) and UDP-glucuronyltransferase (UGT) activity via 4-methylumbelliferyl glucuronide (4-MUG) production (c) showed good cell function compared to cells in a confluent 2D monolayer (dotted lines).

in the gel-free 3D-μFCCS also demonstrated higher UGT activity, producing 0.47–0.63 pmol 4-MUG per million cells (Fig. 5c), as compared to 0.21 pmol 4-MUG produced by a confluent 2D monolayer (dotted line, Fig. 5c). The proliferation and maintenance of good cell functionality of C3A cells in the gel-free 3D-μFCCS throughout the 5-day culture demonstrated the reliability of the gel-free 3D-μFCCS for culturing and maintaining cellular functions in 3D.

To evaluate the gel-free 3D-μFCCS for the culture of sensitive primary cells, we investigated the differentiation competence of the BMSCs by differentiating them down the osteogenic lineage. BMSCs in the gel-free 3D-μFCCS were perfused with osteogenic induction medium for 1 week. von Kossa staining for calcium deposits was positive for BMSCs aggregates in the gel-free 3D-μFCCS (Fig. 6a), similar to von Kossa staining observed in standard confluent 2D cultures after a 1 week of osteogenic induction (Fig. 6b). This suggests that the gel-free 3D-μFCCS may be useful for the culture and study of sensitive primary cells such as the bone marrow-derived adult stem cells.

4. Discussion

We present here a novel method for 3D perfusion cell culture of mammalian cells in microfluidic channels without the use of hydrogels. This gel-free 3D microfluidic cell culture system (gel-free 3D-μFCCS) utilizes inter-cellular polymeric linker to *in situ* form 3D multi-cellular aggregates in microfluidic channels, which are then confined by a micropillar array. The inter-cellular linker acts as a "cell glue" to stabilize the multi-cellular aggregates, such that the cells are supported three-dimensionally by neighboring cells. Compared to microfluidic systems that utilize hydrogels for 3D cell embedment [12,17,22], the use of inter-cellular linker to effect 3D cell culture in the gel-free 3D-μFCCS facilitates the establishment of a more natural extracellular matrix (ECM) environment. This is because the polymeric inter-cellular linker is transient (*i.e.* gradually disappears from the cell surfaces with a half-life of ~2 days), thus allowing cells to secrete and remodel their own ECM environment for maintaining their 3D structural integrity and morphology [28]. The presence of an ECM environment endogenous to a specific cell type in the gel-free 3D-μFCCS may promote more *in vivo*-like cellular phenotypes when compared to microfluidic systems incorporating exogenous ECM hydrogels since cellular phenotypes are highly dependent on the chemical and mechanical properties of the ECM [14].

From an operational point of view, seeding and forming a 3D cell construct in the gel-free 3D-μFCCS is simple. Cells are chemically modified, suspended with the inter-cellular linker, and introduced into the microfluidic channel; the inter-cellular linker induces the formation of 3D multi-cellular aggregates, which are then confined by the micropillar array within the channel. After the central cell compartment of the channel is filled with cells, the cell reservoir is closed; and culture medium is immediately perfused, completing the cell seeding process. Where hydrogels are used to provide 3D cellular support in other microfluidic systems, additional steps are required to induce *in situ* matrix gelation within the microfluidic channel either by photo-polymerization [31], self-assembly of oligopeptides [17], hydrodynamic focusing [17], or polyelectrolyte complex coacervation [13,23]. These steps are more time-consuming and/or require the use of additional fluidic components, such as valves, pumps and connectors to control the delivery of different reagents (Supplementary Fig. S1). In some hydrogel-based systems, suspension of cells in hydrogels is loaded into open microfluidic channels while sealing of the channel is carried out only after the hydrogel has solidified [20,22]. Such operational complexity increases the likelihood of contamination and failure and poses additional challenges for multiplexing design and operation which can be alleviated by using the inter-cellular linker in place of the hydrogels for 3D cellular support.

We demonstrated the utility of the gel-free 3D-μFCCS by successfully culturing and maintaining the 3D phenotypes of model anchorage-dependent mammalian cells, which included carcinoma cell lines (A549 and C3A) and primary bone marrow mesenchymal stem cells (BMSCs). These cell types represent biologically relevant 3D *in vitro* models that can be established with the gel-free

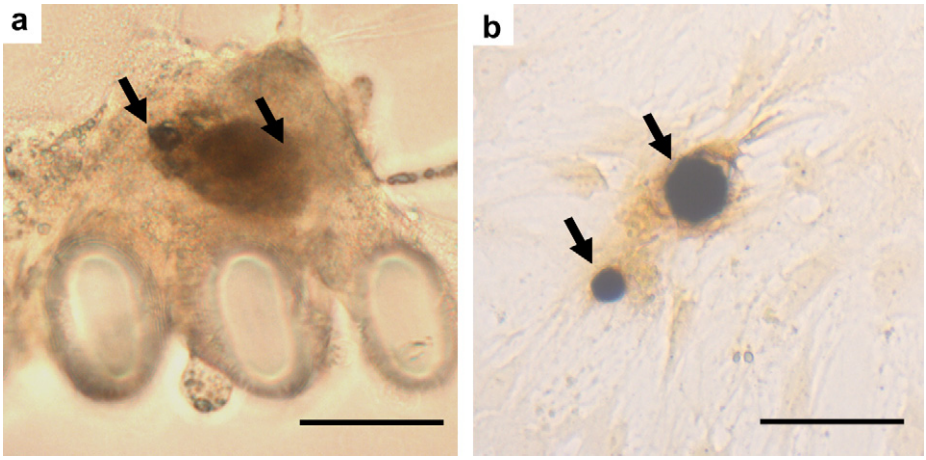

Fig. 6. Primary bone marrow mesenchymal stem cells (BMSCs) can be differentiated into osteoblasts in the gel-free 3D-μFCCS after 1 week of osteogenic induction. (a) von Kossa staining showed calcium salt deposition by BMSCs in the gel-free 3D-μFCCS, similar to the staining obtained in a 2D monolayer control (b). Scale bar: 50 μm.

3D-μFCCS for biological research and drug screening applications. For instance, A549 human lung epithelial cells cultured as 3D cellular aggregates were shown to express increased epithelial cell-specific markers compared to their 2D monolayer counterparts, thus providing a more physiologically relevant model for studying lung infections [32]. C3A human hepatocytes cultured as 3D spheroids exhibited enhanced drug metabolism activities compared to the cells cultured in 2D monolayer, hence providing an efficient experimental tool for predicting drug responses *in vivo* [33]. The successful culture of A549 and C3A carcinoma cell lines in the gel-free 3D-μFCCS shows that it is a good platform for engineering 3D *in vitro* cancer models, in particular, the multi-cellular tumor spheroid (MCTS) model for drug testing applications. The MCTS model is one of the most studied and established 3D cancer models, where cells are held together in 3D by cell–cell interactions, and features key properties of solid tumors such as morphology, cell–cell and cell–matrix interactions, and drug penetration [14,24,25]. The incorporation of 3D cell culture into microfluidic systems would benefit drug screening efforts, as increasing evidence shows that cancer cells cultured in 3D resemble tumors *in vivo* more closely than cells cultured in 2D [34]. While there exists a few microfluidic systems that can support carcinoma cell lines in 3D, they are unable to recapitulate the multi-cellular tumor spheroid (MCTS) model as the cells are embedded in hydrogels [13,17,22]. In comparison, the gel-free 3D-μFCCS is able to form MCTS-like cell-dense constructs with cell–cell contacts (Fig. 4). MCTS-based drug testing can thus be performed in a miniaturized and higher throughput manner with the gel-free 3D-μFCCS.

The gel-free 3D-μFCCS is also capable of supporting sensitive primary mammalian cells, such as mesenchymal stem cells (Figs. 4 and 6). Stem cells are highly prized in regenerative medicine for their ability to self-renew and differentiate into multiple lineages. Its differentiation fate is influenced by specialized microenvironments or cell niche that remain poorly defined [35,36]. 3D perfusion culture of BMSCs has been demonstrated to provide a more physiological environment than conventional 2D cultures for studying proliferation and differentiation [37]. Microfluidic platforms such as the gel-free 3D-μFCCS offer the potential for manipulating the cell niche by allowing different microenvironmental factors to be tested simultaneously when integrated with gradient generators, and maintaining a constant cell microenvironment with one-pass perfusion [30,38]. The biological versatility and simple operation of the gel-free 3D-μFCCS make it a suitable platform to be further adapted for biological research and drug screening applications, enabling the acquisition of *in vitro* data that are more predictive of *in vivo* responses in a high content and high throughput approach.

5. Conclusion

We have developed a gel-free microfluidic cell culture system (3D-μFCCS) that supports 3D perfusion culture of various anchorage-dependent mammalian cells by the combined use of transient inter-cellular polymeric linker and micro-fabricated pillar arrays. The gel-free 3D-μFCCS exhibits a cell-dense construct and endogenously created extracellular microenvironment. We demonstrated the biological versatility of the gel-free 3D-μFCCS by culturing anchorage-dependent mammalian cell lines (A549 and C3A) and primary bone marrow mesenchymal stem cells (BMSCs). The cells showed good cell viability and preserved 3D cell morphology, exhibited higher cell functionality over cells cultured in 2D monolayer, and displayed differentiation capability. The gel-free 3D-μFCCS will be a valuable tool for biological research and drug screening applications.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2008.04.022.

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