Biomaterials 35 (2014) 259–268

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Polymer-based mesh as supports for multi-layered 3D cell culture and assays

Karen A. Simon [a], Kyeng Min Park [a, 1], Bobak Mosadegh [a, b, 1], Anand Bala Subramaniam [a, 1], Aaron D. Mazzeo [a], Philip M. Ngo [a], George M. Whitesides [a, b, *]

[a] Department of Chemistry & Chemical Biology, Harvard University, Cambridge, MA 02138, USA
[b] Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, MA 02138, USA

ARTICLE INFO

Article history:
Received 4 July 2013
Accepted 13 September 2013
Available online 2 October 2013

Keywords:
ECM (extracellular matrix)
3D tumor model
Oxygen gradient
Surface modification
Composite material
Hydrogels

ABSTRACT

Three-dimensional (3D) culture systems can mimic certain aspects of the cellular microenvironment found *in vivo*, but generation, analysis and imaging of current model systems for 3D cellular constructs and tissues remain challenging. This work demonstrates a 3D culture system—Cells-in-Gels-in-Mesh (CiGiM)—that uses stacked sheets of polymer-based mesh to support cells embedded in gels to form tissue-like constructs; the stacked sheets can be disassembled by peeling the sheets apart to analyze cultured cells—layer-by-layer—within the construct. The mesh sheets leave openings large enough for light to pass through with minimal scattering, and thus allowing multiple options for analysis—(i) using straightforward analysis by optical light microscopy, (ii) by high-resolution analysis with fluorescence microscopy, or (iii) with a fluorescence gel scanner. The sheets can be patterned into separate zones with paraffin film-based decals, in order to conduct multiple experiments in parallel; the paraffin-based decal films also block lateral diffusion of oxygen effectively. CiGiM simplifies the generation and analysis of 3D culture without compromising throughput, and quality of the data collected: it is especially useful in experiments that require control of oxygen levels, and isolation of adjacent wells in a multi-zone format.

1. Introduction

The field of tissue engineering has two major motivations: (i) to grow cells in constructs for replacement of organs, and (ii) to create experimental models of tissues (and, ultimately of organs and animals) for *in vitro* studies (e.g., in drug development, toxicology, pharmacokinetics, and radiation biology) that replace more expensive and more complex *in vivo* models [1]. 3D-culture models (organ slices [2], cellular spheroids [3–5], cells seeded or embedded in extracellular matrix (ECM) [6], cells grown in decellularized tissue scaffolds, artificial skin, microcarrier cultures [7])—with appropriate design—can mimic certain aspects of the native microenvironment of cells that can be difficult, if not impossible, to mimic in conventional 2D-culture systems [8]. These 2D-cultures lack a number of essential features required to mimic 3D tissues: i) 3D cell-cell and cell-ECM contacts that affect differentiation of cells; ii) 3D structural features that determine the mass transport-limited rates of molecules (e.g., oxygen, glucose, and carbon dioxide) crucial to the metabolism and viability of cells; iii) 3D stromal tissues that support epithelial cells; (iv) 3D stratification of cells that enables co-culture and interaction of heterogeneous populations of cells; and (v) 3D mechanical stress that regulates behavior of cells in tissues (e.g., bone formation, wound healing, etc) [9–11].

In most tissues, cells are within a distance of 100–200 μm from a blood vessel, and receive sufficient oxygen and glucose by passive diffusion from capillaries to maintain their metabolism [12,13]. Beyond this distance, cells receive amounts of nutrients and molecules (oxygen in particular) that are too limited to allow normal, dioxygen-based metabolism [14,15] and gene expression [16] that influence or determine progression of disease [16,17]. Cancer cells that populate the hypoxic (and often necrotic) central regions of the solid tumors, for example, exhibit stem cell-like properties, and resist both chemotherapy and radiotherapy [18].

Although current 3D cell culture systems allow monitoring of cellular response to different cues (for example, drugs, hormones, signaling molecules, nutrients and toxins, either in uniform concentrations or distributed in gradients in space and time); challenges in

* Corresponding author. Department of Chemistry & Chemical Biology, Harvard University, Cambridge, MA 02138, USA. Tel.: +1 617 495 9430; fax: +1 617 495 9857.
E-mail address: gwhitesides@gmwgroup.harvard.edu (G.M. Whitesides).
[1] Contributed equally.

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.09.049

sample handling [1] and imaging [19] hinder the wide-spread use of these 3D-culture systems. Biological samples with moderate thickness, including cells cultured in 3D (<1 mm thick) are commonly imaged using confocal microscopy [20]. Imaging by confocal microscopy, however, can be challenging because of limitations in optical depth of penetration, and photobleaching of dyes [19,20]. Techniques that either modify the optics of the microscope (e.g., two-photon and multi-photon microscopy) [21–24], or acquire images of the samples from multiple angles (e.g., optical coherence (OCT) [25] and optical projection tomography (OPT)) [26], have been developed to overcome these limitations. These techniques, although successful in increasing the penetration depth of light into the samples, often sacrifice depth of field for resolution. Single (or selective) plane illumination microscopy (SPIM), which combines optical sectioning and tomography with confocal imaging, allows imaging of large samples at high resolutions and with minimal photobleaching [27]; but sample handling can be difficult, particularly with respect to the spatial control of the components within the thickness of 3D-culture models. Deisseroth and co-workers introduced a preparative technique called CLARITY to transform intact tissues into optically-transparent and molecularly-permeable constructs while preserving the native structure of these tissues. This method permits visualization of neurites over long distances and provide information on the topological morphology of traced neurons—information which is lost if specimens of the brain were sectioned mechanically [28].

Recently, we demonstrated that growing mammalian cells in thin (100–200 μm) slabs of paper-reinforced gel ("**C**ells-**i**n-**G**els-**i**n-**P**aper" or CiGiP), provided an experimentally simple approach with which to conduct *in vitro* 3D cell culture [29]. Hydrophobic patterns of wax were printed in arrays across the full thickness of cellulose paper to generate 96 hydrophilic zones that confined cells in circular slabs of ECM-based gels in the paper [30]. By stacking and de-stacking (e.g., peeling apart) sheets of cells embedded in hydrogels, CiGiP provided a simple approach for handling and analyzing cell cultures in 3D without requiring specialized equipment (most analysis can be done using a fluorescent gel scanner). The ease of separating stacked sheets of paper is in sharp contrast to the other methods required for analysis in other 3D cultures: microtomes, multi-photon microscopes, optical coherence tomography systems [20,31], and laser-capture microdissection systems [32].

Although CiGiP simplifies handling and analysis of the cultured cells, the cellulose fibers that constitute paper scatter light (Fig. S-1) and prevent high-resolution imaging of cells using an optical microscope. Many high-resolution techniques used routinely to analyze 2D cell cultures – for example, labeling with colorimetric stains to distinguish intracellular composition and structure, and observing cellular morphology through optical microscopy [20] – therefore cannot be applied directly to CiGiP cultures. Another limitation of CiGiP cultures, as described by Derda et al. [30], is that the wax-printed barriers allow dioxygen (O_2), and we presume, other molecules (certainly CO_2, hydrophobic drugs (e.g., calcein-AM, mitomycin C) and perhaps water-soluble molecules such as glucose) to diffuse laterally within the plane of the sheet from the sides of the stacked 3D constructs. The diffusion of oxygen from the sides allowed cells to survive along the rims of the cell culture zones, even in layers that should, in principle, be severely depleted of oxygen [30].

Here we describe a modification of CiGiP– "**C**ells-**i**n-**G**els-**i**n-**M**esh" or (CiGiM) – for generating 3D tissue models in which we replace the paper in CiGiP with an open polymer mesh as a scaffold to support cells embedded in ECM-based gels. Openings in the mesh (Fig. S-1B) allow unimpeded observation of cells by light microscopy, and high-resolution imaging by confocal microscopy.

2. Materials and methods

2.1. Materials

Reagents and chemicals were obtained from Invitrogen unless otherwise indicated. MDA-MB-231 cells, Eagle's Minimum Essential Medium (EMEM), and trypan blue were purchased from American Type Culture Collection (ATCC). Glutamax™ and penicillin-streptomycin solution were obtained from Gibco. Polybrene was acquired from Sigma. Parafilm® was purchased from VWR, and polyethylene(terephthalate) (PET) mesh was purchased from McMaster-Carr. Paraformaldehyde was from Electron Microscopy Sciences.

2.2. Cell culture and transduction

We cultured MDA-MB-231 breast cancer cells as recommended by ATCC in Eagle's Minimum Essential Medium (EMEM) at pH 7.2 with fetal bovine serum (10% (v/v)), 1% Glutamax™ (1% (v/v)), and penicillin-streptavidin (1% (v/v)). We maintained cells as adherent cultures in vented tissue culture flasks at 37 °C and 5% CO_2. To express green fluorescence protein, the cells were transducted by lentivirus (GFP) in the presence of 5 mg/mL polybrene as described previously by Mammoto et al. [33].

2.3. Preparation of multi-zone PET-based mesh sheets

2.3.1. Fabrication of multi-zone mesh sheet

PET-based mesh sheets were placed on a glass plate inside a SPI Plasma Prep II Chamber (Structure Probe Inc., West Chester, PA). We exposed the mesh to air plasma (100 W, ~1500 mTorr) for 30 min on each side. We created the desired pattern in the Parafilm® by pressing a custom-made steel-rule die (Apple Steel Rule Die. Co., Milwaukee, WI) against the film with a pneumatic press (Tippmann Clicker 1500). Adobe Illustrator CS4 files of the design of the steel-rule die are available by request. We sandwiched the Parafilm® decal and mesh between two aluminum plates. Using a hydraulic press with a heated platen (55 °C), we pressed the aluminum plates with sufficient pressure (~5000 psi) for no more than 20 s (Fig. 1A). Upon releasing the pressure from the hydraulic press, we retrieved the multi-zone mesh with Parafilm® barriers generated by forcing the decal into the mesh.

2.3.2. Sterilization of the mesh

We immersed the multi-zone mesh sheets in ethanol (200 proof) for 1 h. Mesh sheets were then air-dried and exposed to UV light inside a laminar flow hood for another hour. Air-dried samples were stored in a dry Petri dish, sealed with a strip of stretched Parafilm®.

2.4. Fabrication of multi-zone paper-based supports

Multi-zone paper-based supports were prepared as described by Derda et al. [30]. In brief, multi-zone designs were drawn in Illustrator CS4 (Adobe) and printed to a sheet of Whatman #114 paper (20 cm × 20 cm) by a wax printer (Phaser 8560DN, Xerox). We baked the wax-printed paper in an oven (150 °C, 2 min) to melt and allow wax to penetrate through the thickness of the paper. The wax-printed paper was cut to multi-zone plates using a laser cutter (Versa Laser-Universal Laser VL-300). We immersed the baked multi-zone paper in ethanol for 1 h to remove the residual wax. The paper was incubated with a fresh solution of ethanol for an additional hour, and air-dried in a laminar flow hood with UV light for 1 h for sterilization.

2.5. Seeding of cells onto multi-zone mesh sheets and multi-zone paper-based supports

We detached the cells from the tissue culture flasks by incubating in a solution of trypsin-EDTA for 5 min and washing in culture media. We resuspended the cells in Matrigel (BD) at a concentration of 4×10^7 cells/mL Matrigel, or diluted them to obtain the desired concentrations to prepare the calibration curve. For all suspensions, we used Matrigel as received without further dilution. While holding the multi-zone mesh with tweezers, we applied the suspension on the hydrophillized zones of the mesh using a micropipette. Using the tip of the micropipette, we spread the suspension on the zone to allow complete permeation of the mesh in the zone by the cells in Matrigel. Unless noted otherwise, we applied 3 μL of the 4×10^7 cells/mL Matrigel suspension on each zone. Based on this volume and concentration, the initial seeding density in each zone was 1.2×10^5 cells/zone. After seeding, we placed the mesh in a Petri dish containing medium that was pre-warmed to 37 °C. To allow the Matrigel to gel completely, we incubated the multi-zone mesh, or paper containing suspensions of cells, in Matrigel for at least 12 h, prior to stacking.

2.6. Culture of stacked CiGiM

To keep the sheets in conformal contact and submerged in culture medium, we sandwiched layers of paper or mesh containing cells embedded in Matrigel in a