Multilayer microfluidic PEGDA hydrogels

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Development of robust 3D tissue analogs in vitro is limited by passive, diffusional mass transport. Perfused microfluidic tissue engineering scaffolds hold the promise to improve mass transport limitations and promote the development of complex, metabolically dense, and clinically relevant tissues. We report a simple and robust multilayer replica molding technique in which poly(dimethylsiloxane) (PDMS) and poly(ethylene glycol) diacylate (PEGDA) are serially replica molded to develop microfluidic PEGDA hydrogel networks embedded within independently fabricated PDMS housings. We demonstrate the ability to control solute-scaffold effective diffusivity as a function of solute molecular weight and hydrogel concentration. Within cell laden microfluidic hydrogels, we demonstrate increased cellular viability in perfused hydrogel systems compared to static controls. We observed a significant increase in cell viability at all time points greater than zero at distances up to 1 mm from the perfused channel. Knowledge of spatiotemporal mass transport and cell viability gradients provides useful engineering design parameters necessary to maximize overall scaffold viability and metabolic density. This work has applications in the development of hydrogels as in vitro diagnostics and ultimately as regenerative medicine based therapeutics.

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1. Introduction

Development of robust, in vivo-like tissues in vitro holds the potential to create regenerative medicine based therapeutics, provide more physiologically significant pre-clinical models and supply a pharmacological and toxicological screening platform that reflects in vivo systems in both complexity and function. Despite significant progress, tissue engineering research has largely failed to produce tissue or organ level systems in vitro. This is thought to be primarily due to difficulty translating systems from 2D to 3D. The most significant challenge when developing 3D systems is the mass transport limitations encountered when scaling up to metabolically dense constructs over clinically relevant length scales.

The most significant mass transport limitation of tissue engineered constructs is the reliance on passive diffusion of nutrients to sustain cellular metabolic activity. Passive diffusion inadequately delivers nutrients over large length scales and limits the size and metabolic density of the construct. Furthermore, build up of nutrient and waste gradients within the construct reduces cell viability and disrupts cellular phenotype [1]. Inadequate transport of nutrients and waste has been correlated with a decrease in tissue quality as a function of distance from the nutrient source with the most distant regions becoming metabolically inactive or necrotic [2,3]. Due to these limitations, much effort in the fabrication of tissue engineering scaffolds has focused on enhancing the mass transport properties of the construct to maximize cellular viability and modulate signaling gradients for the control of cellular behavior and tissue formation.

A wide variety of scaffold fabrication techniques has been developed to alter scaffold bulk properties in order to improve mass transport and cellular migration characteristics. Particulate leaching [4], polymer phase separation [5], gas foaming [6] and electrospinning [7] have all been shown to increase average pore size, facilitate pore interconnectivity, and enhance overall porosity at the nano- and micro-scale. Although these techniques have been shown to improve bulk mass transport properties, they provide little or no spatial control over fabricated microarchitecture. Furthermore, cytotoxic processing conditions require cell seeding to occur post scaffold processing. This methodology often results in non-homogenous cellular distribution throughout the thickness of the scaffold and limits the overall metabolic density of the construct [8–10].

In order to improve upon these scaffold processing limitations, microfabrication techniques have been employed to improve mass transport properties in tissue engineering scaffolds. These techniques allow for spatial patterning of the microarchitecture and control over spatiotemporal mass transport gradients while maintaining the ability to homogenously encapsulate cells within the scaffold bulk.
Direct photolithographic patterning techniques have been used to modify micro-scale biochemical, biomechanical and diffusion properties [11–13], photodegrade crosslinks to form channels through direct laser irradiation, [14,15] and to build microarchitectures via sequential additive photopatterning methods [16].

Perhaps the most significant advancement to improve scaffold mass transport properties is the fabrication of microfluidic networks in tissue engineering constructs using soft lithographic replica molding techniques. These networks have been used to facilitate convective and diffusive mass transport [17], spatiotemporally introduce reagents into the scaffold bulk from perfused channels, [18] and direct cell localization within the microchannel lumen [19] and the scaffold bulk [20,21]. Most recently, multilayer microfluidic devices have been developed combining poly(dimethylsiloxane) (PDMS) and hydrogel microarchitectures to study cell migration [22] and spatially control microenvironment properties within perfused channels [23].

Despite significant advances in microfabricated tissue engineered scaffolds, application of microfabrication techniques to photopolymerizable synthetic hydrogel materials has remained absent from the literature. The inherently weak mechanical properties of microfluidic hydrogels require complex multilayer systems to support cell proliferation (Choi et al., in preparation) [23,24]. Furthermore, these complex systems lack independent control over scaffold and microchannel network geometry which greatly reduces the flexibility to adjust system design. Moreover, network design has largely ignored the need to maximize construct metabolic density by optimizing microfluidic network parameters such as channel spacing. We report a simple, robust, multilayer replica molding technique in which PDMS and poly(ethylene glycol) diacrylate (PEGDA) are serially replica molded to develop microfluidic PEGDA hydrogel networks embedded within independently fabricated PDMS housings. This technique allows for the production of perfusable PEGDA networks independent of overall scaffold geometry. Furthermore, the mild PEGDA photocrosslinking conditions permit homogeneous and high density cellular encapsulation [16,24]. In addition, we show the ability to quantify and modulate spatial temporal mass transport properties of the construct by varying hydrogel concentration and perfused solute molecular weight. Finally, we demonstrate the ability to sustain encapsulated cellular elements for 72 h in culture and determine the optimal microfluidic vessel spacing to maintain cell viability and maximize construct metabolic density. Additionally, the application of these techniques to photopolymerizable PEGDA hydrogel materials provides the unique ability to photolithographically pattern biochemical, biomechanical and hydrogel physical architectures independent of the soft lithographic fabrication steps reported herein. Together these techniques represent a significant contribution towards the development of metabolically dense, complex 3D tissue analogs in vitro.

2. Methods and materials

2.1. Multilayer microfluidic device fabrication

2.1.1. Fabrication of SU-8 photore sist masters

Glass slides were cleaned and hydroxylated in piranha solution (7:3 v/v solution of H2SO4, 30% H2O2) for 30 min and dehydrated for 15 min at 200 °C prior to spin coating. SU-8 2100 (Microchem) was spun coat at 1000 rpm for 40 s to generate a 350 µm thick substrate and SU-8 2050 (Microchem) was spun coat at 1000 rpm for 40 s to generate a 100 µm thick substrate. Photore sist substrates were exposed via a Mask Aligner (SUSS) with 20,000 dpi transparency masks (CAD/ART Services). All other photore sist processing parameters were derived from manufacturer recommended protocols. 350 µm photore sist masters used to mold the PDMS housings, were cleaned with 100% ethanol, dried with filtered air, and molded to PDMS. 100 µm photore sist masters used to mold the PEGDA microchannels were cleaned with 100% ethanol, dried with filtered air and coated with SigmaCote (Sigma) to prevent adhesion of PEGDA structures to photore sist masters.

2.1.2. PDMS housing fabrication

PDMS (Sylgard 184) was mixed (10:1; silicone elastomer:curing agent), degassed, and cured to 350 µm photore sist masters for 3 h at 65 °C (Fig. 1, Step 3). PDMS housings were removed from the photore sist master, access ports were bored with a 1 mm biopsy punch, and polyethylene tubing was inserted into the access ports and sealed through compression fitting. All PDMS housings were treated with the photoinitiator acrytophenone (2,2-dimethoxy-2-phenyl acrylophenone in n-vinylpyrrolidone, 300 mg/mL Sigma) for 5 min to promote free radical induced interfacial polymerization of the PEGDA pre-polymer solution to the PDMS housing. Excess photoinitiator was removed via sequential rinsing with 100% ethanol and ultrapure H2O and dried with filtered air.

2.1.3. Fabrication of multilayer microfluidic hydrogels

Acetophenone coated PDMS housings were aligned and overlaid with the SU-8 2050 photore sist masters treated with SigmaCote. PEGDA pre-polymer solutions containing cells or PEGDA alone were injected into the housing around the channel mold and exposed to a long wavelength UV lamp (365 nm, 10 mW/cm2) for 2.5 min (Fig. 1, Step 5). In order to form 3D microfluidic networks, acetophenone coated PDMS housings were sealed against a flat substrate and PEGDA pre-polymer solutions were injected into the housing and under crosslinked via a 1 min UV exposure to form a blank slab (Fig. 1, Step 8). The PDMS/PEGDA microchannel mold and the under exposed blank slab were brought into contact and exposed to UV for 2.5 min to graft the hydrogel blank slab to the hydrogel with channel in relief (Fig. 1, Step 9). For all experiments reported herein, the PDMS housing with an embedded PEGDA microfluidic hydrogel was conformally sealed to coverglass, and polyethylene perfusion tubes were connected to the inlet reservoir and outlet syringe pump. Perfusion was initiated at 600 µL/h using a syringe pump (Harvard Apparatus) in withdrawal mode.

2.2. Cell maintenance

NIH 3T3 fibroblasts, passages 3–10, (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma) supplemented with 10% (v/v) heat inactivated fetal bovine serum (PBS; BioWhittaker, Walkersville, MD, USA), 2 mm L-glutamine, 100 U penicillin, and 100 µg/mL streptomycin at 37 °C in 5% CO2 to 90% confluence prior to seeding.

2.3. Preparation of PEGDA pre-polymer solution for spatiotemporal transport studies

6 kDa PEGDA was synthesized according to previously published methods [12]. Briefly, PEGDA with a molecular weight (Mw) of 6000 g/mol was prepared by combining 0.1 mmol/mL dry poly(ethylene glycol) (Fluka), with 0.4 mmol/mL acryloyl chloride, and 0.2 mmol/mL triethylamine in anhydrous dichloromethane (DCM, Sigma) and stirred under argon overnight. The resulting solution was washed with 2 M HCl, and separated into aqueous and DCM phases to remove hydrochloric acid. The DCM phase was subsequently dried with anhydrous MgSO4 (Sigma), and PEGDA was precipitated in diethyl ether (Fisher), filtered, and dried under vacuum.

2.4. Preparation of PEGDA pre-polymer solutions for spatiotemporal transport studies

Solutions were prepared containing 6 kDa PEGDA in PBS (pH 7.4) to yield final concentrations of 5, 10 or 20% (w/v). Igracure 2959 (Ciba, 100 mg/mL in 100% ethanol) was added to the hydrogel pre-polymer solution at 30 µL/mL.

2.5. Preparation of PEGDA pre-polymer solution and acryloyl-PEG-RGDS for spatiotemporal cell viability studies

6 kDa PEGDA was dissolved in sterile PBS (pH 7.4) to yield a final concentration of 10% (w/v), 3 mm acryloyl-PEG-RGDS, synthesized according to previously reported methods [25] was added to the pre-polymer solution to allow for cell adhesion and promote cell cycle progression. NIH 3T3 fibroblasts were trypsinized, counted and suspended within the PEGDA pre-polymer solution at 11 × 10^6 cells/mL. The cell/ PEGDA pre-polymer solution was replica molded to yield a microfluidic hydrogel as described in Section 2.1.3. Cylindrical cell laden (11 × 10^6 cells/mL) hydrogel constructs, 3 mm in diameter and height, were placed in well plates containing 1 mL of DMEM and used as static controls. Encapsulated cells were cultured for 0–72 h under perfused or static conditions.

2.6. Spatiotemporal diffusion profile analysis

Immediately post device fabrication and sealing to coverglass, acellular PDMS/PEGDA microfluidic hydrogels were perfused with model transport molecules at various molecular weights (305 Da toluidine blue, 3 kDa dextran fluorescein, or 10 kDa dextran fluorescein). Images of the spatiotemporal fluorescent diffusion profiles were taken at 0, 1, 2, 4, 8, and 12 h post perfusion using a fluorescent microscope (Zeiss Discovery V 8 Stereo Scope, 1×, EXPLO X-cite light source) and a color CCD camera ( Jenoptik, 5 megapixel, 16-bit). Images were analyzed using ImageJ (NIH) and spatial intensity profiles were generated using the plot profile function to determine spatial fluorescent intensity at each time point. Intensity
Fig. 1. Fabrication schematic of PDMS/PEGDA microfluidic networks. Serial replica molding of PDMS and PEGDA to photoresist masters produces a perfusable PEGDA microfluidic channel embedded within an exterior PDMS housing. The PDMS/PEGDA construct, Mold A, can be sealed to coverglass for imaging after step 5 or crosslinked to a PEGDA blank slab, Mold B, to produce a 3D microchannel environment (Step 9).
profiles were normalized to maximum intensity and plotted as a function of distance from the microchannel wall, perfusion time, and molecular weight of the transport molecule. Effective diffusivity of the hydrogel–solute system was determined by iterative parameter fitting of the measured spatiotemporal diffusion profiles to the solution of a one-dimensional, non-steady state, first order Fickian diffusion equation using Igor Pro (WaveMetrics) software package (see Supplementary material). Effective diffusivity as a function of solute molecular weight in water was determined using the Stokes–Einstein relationship [26] (see Supplementary material).

2.7. Spatiotemporal cell viability analysis

Spatiotemporal cell viability profiles were obtained at 0, 24, 48, and 72 h post encapsulation at regions ranging from 0 to 1500 μm from the perfused microchannel or 0–1500 μm from the edge of the static tissue plug. Cell viability was assessed using a Live/Dead assay (Calcein AM/Ethidium homodimer, Invitrogen). The viability staining was imaged using a confocal microscope (Carl Zeiss Inc. LSM 5 Live, 20× (NA 0.8) objective, FOV = 318 × 318 μm, vertical z-stack: 1 μm slices, 50 slices per region). Images were tiled outward from the microchannel wall at 300 μm intervals between 0 and 1500 μm to determine cellular viability as a function of distance from the microchannel (Fig. 5, B). At each region the ratio of live cells (Calcein AM positive) to total cells (Calcein AM + Ethidium homodimer) was analyzed as a function of distance from channel and culture time.

3. Results and discussion

3.1. Fabrication of multilayer microfluidic hydrogels

Here we describe a multilayer soft lithographic fabrication technique wherein PDMS and PEGDA were serially replica molded to form a microfluidic PEGDA hydrogel embedded within an external PDMS housing. Serial replica molding of PDMS followed by PEGDA formed robust, perfusable hydrogel microchannel networks.
with channel diameters ranging from 50 to 500 μm (Fig. 2). This fabrication process, along with the rapid and mild photocrosslinking conditions utilized for the PEGDA hydrogel formation, allowed for encapsulation of cells within the hydrogel matrix with minimal cytotoxicity (Figs. 5 and 6, t = 0 h). The development of perfusable microfluidic hydrogels and complementary photolithographic fabrication methodologies provides a robust tool set to build complex, micro-scale, biomimetic, tissue analogs in vitro.

3.2. Molecular weight effects on spatiotemporal transport gradients

Inadequate nutrient and waste mass transport limits the size, complexity and clinical functionality of tissue engineered constructs. Optimization of nutrient and waste mass transport coupled with control over spatiotemporal signaling gradients provides the ability to maximize cell viability, spatially control cell behavior and direct tissue formation. We demonstrated a microfluidic PEGDA hydrogel system that supports pressure driven convective transport as well as diffusional transport mechanisms. Spatiotemporal molecular gradients were shown to vary as a function of solute molecular weight (Fig. 3) and hydrogel concentration (Fig. 4). As expected, diffusional transport of higher molecular weight solutes with larger hydrodynamic radii occurred at a slower rate when compared to that of lower molecular weight solutes (Figs. 3 and 4). Toluidine blue (M_w = 305 Da) exhibited the highest effective diffusivity (1.75 x 10^{-9} cm^2/s) while 3 kDa dextran fluorescein (1.23 x 10^{-7} cm^2/s) and 10 kDa dextran fluorescein (2.6 x 10^{-9} cm^2/s) exhibited lower effective diffusivities in proportion to their increasing molecular radius (Fig. 4D).

In addition to solute molecular weight effects on spatiotemporal molecular diffusion gradients, PEGDA hydrogel concentration was shown to affect system transport properties (Fig. 4). Increases in hydrogel concentration from 5 to 20% (w/v) were shown to be inversely related to solute diffusion rate and the effective diffusivity of the hydrogel–solute system (Fig. 4D). At the low hydrogel concentration (5%), toluidine blue had the highest effective diffusivity (1.86 x 10^{-9} cm^2/s). However, when increasing the hydrogel concentration to 10 and 20%, the effective diffusivity of toluidine blue decreased to 1.75 x 10^{-9} and 8.45 x 10^{-9} cm^2/s respectively (Fig. 4D). This trend was attributed to the decrease in average hydrogel pore size as a function of increasing PEGDA concentration (see Supplementary material). Modulation of diffusion gradients by varying polymer physical properties and solute molecular weight provides a useful tool to control soluble spatiotemporal signaling gradients and to direct cell migration and tissue pattern formation in vitro.

3.3. Spatiotemporal cell viability gradients

Optimization of nutrient and waste gradients through rational microfluidic network design provides a tool to maintain cell viability and increase overall scaffold metabolic density. Viability of encapsulated 3T3 fibroblasts was shown to vary as a function of culture time, distance from perfused channel, and static vs. perfused culture (Figs. 5 and 6). A trend of decreasing cell viability.
with increasing distance from the microchannel was observed between 24 and 72 h of culture (Figs. 5 and 6). Regions closest to the channel (0–600 μm) were shown to have significantly higher cellular viability than the external regions (600–1500 μm) at both 48 and 72 h of culture (paired t-test, n = 3, p < 0.05). This trend was not observed at early time points and only became pronounced later in the culture (Figs. 5 and 6).

Necrotic region (cell viability <30%) size as well as the overall system viability was improved in perfused systems when compared to static controls (Fig. 6). 3T3 fibroblast viability in perfused systems was significantly higher compared to static controls at all time points greater than zero for distances between 0 and 900 μm from the microchannel (paired t-test, n = 3, p < 0.05) (Fig. 6). Necrotic regions in static systems at late time points (t = 72 h) were shown to occur throughout the entire thickness of the scaffold, while necrotic cores in perfused systems were confined to the outermost regions of the scaffold (distances >600 μm; Fig. 6). Increased cell viability in perfused systems was attributed to convection-driven renewal of nutrient concentration gradients that are otherwise depleted over time in static systems. Furthermore, removal of waste products that otherwise accumulate in static systems was thought to also positively affect cell viability.

Spatiotemporal cellular viability profiles closely reflected conclusions derived from models which assume that cells act as metabolic sinks with intrinsic properties such as metabolic consumption rate and extrinsic factors such as cell seeding density [2,27,28]. These assumptions allowed cell laden hydrogel scaffold systems to be modeled as having an initial average metabolic density defined as the product of cellular metabolic consumption rate and cell seeding density. However, when scaffold metabolic densities exceeded the rate at which critical solutes are delivered to the system, inadequate nutrient and cytotoxic waste gradients develop and cellular elements become quiescent and/or apoptose. This transport regime results in metabolic density profiles that parallel cell viability and ultimately generate a construct with reduced overall metabolic density. A detailed understanding of metabolic gradients as a function of cell type and seeding density provides useful rational design parameters, such as channel spacing, that allows for maximization of cellular viability and scaffold metabolic density. For example, a channel spacing of 600 μm for 3T3 cultures seeded at 11 × 10^6 cells/mL would lead to greater than 60% average construct viability which is nearly a six fold increase over the static system (Fig. 6). In order to maximize...
scaffold metabolic density, careful attention must be paid to design mass transport regimes that minimize necrotic regions and conserve the regenerative potential of the construct.

4. Conclusions

In summary, we have demonstrated a multilayer soft lithographic fabrication scheme in which PDMS and PEGDA were serially replica molded to form enclosed perfusable microfluidic hydrogel networks. We demonstrated the ability to control spatiotemporal solute diffusion profiles within the system by varying hydrogel concentration and solute molecular weight. In addition, we show enhanced cellular viability within the perfused microfluidic hydrogel when compared to static controls. Optimization of signaling gradients and cell viability provides the ability to spatiotemporally control the soluble cell microenvironment and maximize construct metabolic density. This work has direct applications towards the development of robust, complex, cell-laden hydrogels for in vitro diagnostics and regenerative medicine applications.

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Appendix. Supplementary material

Supplementary material associated with this article can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.03.031.

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