A 3D-printed microbial cell culture platform with *in situ* PEGDA hydrogel barriers for differential substrate delivery

Andrea L. Kadilak,¹ Jessica C. Rehaag,¹ Cameron A. Harrington,¹
and Leslie M. Shor^{1,2,a)}

¹Department of Chemical and Biomolecular Engineering, University of Connecticut, Storrs, Connecticut 06269-3222, USA

²Center for Environmental Sciences and Engineering, University of Connecticut, Storrs, Connecticut 06269-4210, USA

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Additive manufacturing, or 3D-printing techniques have recently begun to enable simpler, faster, and cheaper production of millifluidic devices at resolutions approaching 100–200 μm . At this resolution, cell culture devices can be constructed that more accurately replicate natural environments compared with conventional culturing techniques. A number of microfluidics researchers have begun incorporating additive manufacturing into their work, using 3D-printed devices in a wide array of chemical, fluidic, and even some biological applications. Here, we describe a 3D-printed cell culture platform and demonstrate its use in culturing *Pseudomonas putida* KT2440 bacteria for 44 h under a differential substrate gradient. Polyethylene glycol diacrylate (PEGDA) hydrogel barriers are patterned *in situ* within a 3D-printed channel. Transport of the toluidine blue tracer dye through the hydrogel barriers is characterized. Nutrients and oxygen were delivered to cells in the culture region by diffusion through the PEGDA hydrogel barriers from adjacent media or saline perfusion channels. Expression of green fluorescent protein by *P. putida* KT2440 enabled real time visualization of cell density within the 3D-printed channel, and demonstrated cells were actively expressing protein over the course of the experiment. Cells were observed clustering near hydrogel barrier boundaries where fresh substrate and oxygen were being delivered *via* diffusive transport, but cells were unable to penetrate the barrier. The device described here provides a versatile and easy to implement platform for cell culture in readily controlled gradient microenvironments. By adjusting device geometry and hydrogel properties, this platform could be further customized for a wide variety of biological applications. Published by AIP Publishing. [<http://dx.doi.org/10.1063/1.5003477>]

I. INTRODUCTION

Implementations of additive manufacturing approaches for the production of microfluidic and millifluidic devices are increasingly common due to rapid improvements in performance and affordability. Producing millifluidics *via* 3D-printing is simpler, cheaper, and faster than photolithography with soft lithography; desktop 3D printers can be purchased from between \$2000–5000, and the entire design and production of devices takes days as opposed to weeks or months with traditional photolithography and soft lithography techniques.^{1–5} 3D-printing may also have superior capabilities for some applications. For example, 3D-printed devices have been fabricated with complex shapes like spirals, corkscrews, and grooved surfaces to improve mixing;^{1,6,7} such shapes cannot be produced by photolithography. 3D-printed micro-reactors with in-line product-testing have been developed for both organic and inorganic

^{a)} Author to whom correspondence should be addressed: leslieshor@gmail.com. Tel.: (860) 486-3136.

reactions.^{3,6} Recently, printed pneumatic valves have been used to control flow configurations.^{8,9} Researchers have also begun using 3D-printed microdevices for biological applications. Typical feature sizes of 3D printed devices can be ideal for certain biological applications where microfluidics are too small but liquid volumes of conventional cell culture methods are too large. Common biological applications for 3D printed devices include cell separation¹⁰ and screening assays.¹¹ For example, sensor devices have been developed to detect *Staphylococcus aureus* in environmental samples¹² and for measuring changes in cell growth with antibiotic administration.^{13,14}

Stereolithography (SLA) printers are often used for producing 3D-printed millifluidics because they can achieve higher resolutions than fused deposition modeling (FDM) printers and also readily achieve mechanically stable, fully enclosed channels.^{10,11} This is because FDM printers work by extruding and layering filaments of melted polymer or plastic onto the build platform; the resolution is determined by the size of the extruded filament and the melted polymer requires some time to solidify, making unsupported “ceilings” of an enclosed channel prone to collapse.^{10,11} Meanwhile, STL printers work by focusing UV lasers point by point in the desired pattern to solidify photo-curable acrylate or methacrylate-based polymer resins.^{10,11,15} This process is repeated for each layer, and the resolution is determined by the size of the laser point and the molecular weight of the polymer. Because the polymer cures and solidifies immediately upon UV exposure with the laser, enclosed channel structures printed using STL are more mechanically stable.

However the photo-curable acrylate or methacrylate-based polymer resins and photoinitiators used in SLA printers may be toxic to cells that are in contact with them for an extended period of time.^{2,16,17} To mitigate SLA resin biotoxicity, prior work has limited the time of cell contact with the resin^{10,12} or incorporated membranes or other barriers to prevent direct contact between cells and SLA resin.¹⁴ Recently, researchers working with zebrafish embryos as a model organism reported a diminution in toxicity effects with certain resin pre-treatments, notably additional UV exposure and washing in water or ethanol.^{18–21} In the work described here, we implemented the most successful pre-treatment methods described in the literature and followed the manufacturer’s recommendations to improve resin biocompatibility. By following these steps, and by selecting a robust bacterium known tolerate and even biodegrade environmental contaminants,^{22,23} we hypothesized that we could successfully culture bacteria within a 3D-printed channel over an extended period of time.

One important application of microfluidic and millifluidic devices is to study cellular function *in situ* within realistic microenvironments.^{24,25} Microbes living in natural microenvironments typically experience chemical gradients at the micrometer to millimeter scale. For example, microbial communities in soil, in the human gut, or flocs in a wastewater treatment plant experience steep changes in the oxygen concentration with position. In contrast, traditional bulk culturing techniques tend to be well-mixed, and expose relatively large cell populations to a single substrate concentration, oxygen level, and/or pH. In order to better understand cellular function, it is important to perform cell culture under realistically non-uniform chemical conditions.²⁶

The typical approach for culturing cells within a chemical gradient is to expose opposing edges of a central culture chamber to different substrates or buffer mixtures. Variations on this basic fluidic design have been employed for culture of stem cells,²⁷ cancer cells,^{28,29} immune cells,²⁹ epithelial cells,³⁰ white blood cells,³¹ and bacteria.³² In many cases, cells in the central culture chamber are immobilized in a hydrogel so flow from the perfusion channels do not flush out the culture chamber and/or motile cells do not exit the culture chamber. Saadi *et al.*³³ used the basic cell culture channel design described above and incorporated hydrogels to establish a chemical gradient and study the chemotaxis of red blood cells in response to that gradient. Their ladder-like device consists of two parallel microfluidic flow channels connected by capillaries filled with collagen hydrogel. In their design, the relatively small surface area and long length of the capillaries could lead to relatively low diffusive flux and long equilibration times, which may be undesirable for some applications. In contrast, the cell culture platform described here offers a scaled up version of the simple gradient chamber design with hydrogel

barriers enabling higher fluxes and more efficient substrate delivery to cells. In addition, cells do not need to be immobilized in a hydrogel, enabling studies using motile cells focusing on cellular responses in liquid environments.

Hydrogels have been used in cell culture systems for many years and are widely used in biomedical,^{34,35} biomolecular,³⁶ microbiological,^{37–41} and drug delivery^{42,43} applications. In micro- or millifluidic systems, hydrogels are frequently employed to immobilize or sequester cells and/or to prevent fluid flow. Hydrogels are a family of hydrophilic polymers that form a cross-linked network in an aqueous solution.^{42,44,45} The elastic property of hydrogels further increases resistance to flow compared with liquids,^{44,46} yet hydrogels have diffusive transport properties similar to liquid water when in their hydrated state.⁴⁶ Many microfluidic devices exist that utilize the diffusive properties of biocompatible hydrogels, such as agar, agarose, and gelatin, to develop stable concentration gradients in cell culture applications.^{32,47–53} In addition, synthetic polyethylene glycol (PEG)-based hydrogels are widely used in microfluidics because PEG is biocompatible and can be patterned *in situ* within a channel by applying UV to a specified area.^{38,39,54–60} With photoactive PEG species such as polyethylene glycol methacrylate (PEGMA) and polyethylene glycol diacrylate (PEGDA), the active acrylate and methacrylate groups interact with one another to form a cross-linked network. The extent of cross-linking or polymerization can be varied to control diffusive properties of the hydrogel *via* size exclusion from the networked mesh or side chain properties, while resisting convective mixing within a microfluidic system.³⁷

Here we describe the construction of a 3D-printed gradient cell culture platform. Our approach enables higher flux, faster equilibration, and simpler fabrication than prior reports. Further, the cell population is not immobilized, so growth and reorganization can be observed in real time in a liquid environment as a function of substrate delivery. Features include PEGDA barriers selectively photopatterned *in situ* within the 3D-printed device that allow for diffusive delivery of nutrients and oxygen to cells. The transport properties of the PEGDA barrier were characterized by comparing observed diffusion of a tracer dye and with finite element modeling simulations. Substrates from constantly flowing perfusion channels were delivered to cells through PEGDA barriers in a long-term growth experiment. Over the course of 44 h, *P. putida* KT2440 bacteria were successfully grown in the culture chamber and spatial organization of cells near higher concentrations of fresh media and oxygen supplied through the PEGDA barrier was observed and quantified.

II. EXPERIMENTAL MATERIALS AND METHODS

A. Chemicals

Clear Resin GPCL02 (Formlabs, Somerville, MA) was used for all devices. Phosphate buffered saline (PBS) (1× powder concentrate) and isopropanol (IPA) (histological grade) were purchased from Fisher Scientific. Polyethylene glycol diacrylate (PEGDA, molecular weight: 700), toluidine blue tracer dye (for microscopy, histological grade), and 2-Hydroxy-2-methyl-propionophenone (HMPP, 97%) water soluble photoinitiator were purchased from Sigma-Aldrich. Deionized (DI) water (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) was produced from a Direct-Q 3 UV Millipore ultrapure water system.

B. Bacteria strains and culture media

Plasmid pCMB55 was used to enable *Pseudomonas putida* KT2440, a motile soil bacterium, to produce green fluorescent protein (GFP) during all phases of growth by expressing the gene *sfGFP* with the strong promoter *P_{trc}*. *P. putida* KT2440 was provided by the lab of Dr. Daniel Gage at the Molecular and Cell Biology Department at the University of Connecticut, who received it from Victor de Lorenzo of the Spanish National Center for Biotechnology, Madrid, Spain. Plasmid pCMB55 also confers tetracycline resistance. Bacteria were grown in lysogeny broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) for routine maintenance, and 21C medium⁶¹ at a 1× strength was used for overnight culture preparations of cells and for

the substrate supplied for the long-term bacterial growth experiment. Saline solution $1\times$ PBS (81% NaCl, 14% sodium phosphate dibasic, 3% sodium phosphate monobasic, 2% potassium chloride) was used for washing and resuspending cells for the bacterial growth experiment. All broth cultures and microfluidic device inocula contained tetracycline (0.01 mg/ml) for plasmid maintenance and to help prevent contamination from any wild-type bacteria present in the laboratory environment.

C. Microdevice design and 3D-printing

3D-printed devices were designed using Solidworks 2015, a CAD software, then imported into the PreForm software, and printed on a Form 2 stereolithography printer (Formlabs, Inc., Somerville, MA) at a resolution of 0.025 mm. All devices were printed using Clear Resin GPCL02 (Formlabs, Somerville, MA), which is a proprietary blend of methacrylated oligomers, methacrylated monomer, and photoinitiator(s).¹⁵ An uncross-linked resin as supplied by the manufacturer is cross-linked into a hardened plastic device using high intensity UV lasers in the SLA printer; the energy from the UV light excites the photoinitiators within the resin mixture, beginning a chain reaction cross-linking methacrylate groups in the oligomers and monomers.⁶² The devices were printed upside-down at a 30° angle with respect to channel edges to allow uncured resin to drain from hollow spaces over the course of the print. CAD drawings and a photograph of the 3D-printed cell culture platform can be seen in Fig. 1.

The design consists of two flow channels connected to a central culture chamber by perpendicular cross-channels. The flow channels and central culture chamber are 2 mm wide and 20 mm long [Fig. 1(c)]. Perpendicular cross-channels are 2 mm wide and 3 mm long, and all channels have a height of 2 mm. The resolution capability of the printing method and reproducibility of these channel dimensions were confirmed by taking 5 measurements at these locations in 3 identical devices (total $n=15$ for each dimension measured); the channel width was found to be 2.09 ± 0.01 mm, and the hydrogel channel length was measured as 3.16 ± 0.01 mm, showing that the device geometry varied only by approximately $\pm 10\ \mu\text{m}$. The thickness of the printed resin at the bottom viewing face of the channel is 0.5 mm to minimize added opacity when contents of the device were viewed on an inverted microscope. All inlets feature barbed fittings designed to fit snugly with 1/8 in. tubing.

After printing, finished devices were placed in isopropanol (IPA) and each of the channels were cleared of uncross-linked resin by rinsing thoroughly with IPA using a wash bottle. Devices were then soaked in the IPA bath for approximately 30 min to remove any uncrosslinked resin, and rinsed with fresh IPA within channels and on the exterior of the device, per manufacturer instructions, then dried in air with ambient room lighting for a minimum of 16 h. Finally, scaffolding supports were removed. If any remaining uncross-linked resin was observed, channel interiors were once again rinsed with fresh IPA and devices were allowed to dry thoroughly in a chemical fume hood. The bottom viewing surfaces of the devices were wet sanded with 3200, 4000, 6000, and finally 12 000 grit sandpaper (“Water-resistant sandpaper for smoothing and polishing plastics,” purchased from McMaster Carr, Product # 4611A109) and rinsed with MilliQ water. Acrylic cleaner (Novus 1, Plastic Clean and Shine) was next

FIG. 1. (a) CAD design of the 3D-printed culture channel device, (b) photograph of the printed device, and (c) diagram of channel dimensions.

sprayed on a piece of cork and the bottom viewing face of the device was polished then finally cleaned with a microfiber cloth. Any rough edges left by supports on other portions of the device were sanded as needed.

D. Photopatterning of PEGDA barriers *in situ*

PEGDA barriers were cross-linked and photopatterned *in situ* within a 3D-printed channel by adapting methods developed for cross-linking and photopatterning PEGDA *in situ* within polydimethylsiloxane (PDMS) microfluidic channels.^{38,39,57} A 20% v/v solution of PEGDA (MW 700) in water was prepared, and then the water soluble photoinitiator HMPP was added to a final concentration of 0.5% v/v. The PEGDA + HMPP solution was vortexed to mix thoroughly. A cleaned and polished 3D-printed channel device was prepared as detailed in Sec. II C, and the empty device was placed in a plasma cleaner (Model PDC-32G, Harrick Plasma, Ithaca, NY) and exposed to oxygen plasma for 45 s; this process makes the interior channel surfaces hydrophilic and aids in filling the channels with PEGDA solution. After plasma exposure, the channels were immediately filled with 20% v/v PEGDA solution with 0.5% v/v HMPP by snugly fitting a 1 ml micropipettor tip into one of the middle channel outlets and delivering approximately 300–400 μ l of solution. All six channel outlets were then capped. Closures for 3D printed barbed channels were prepared by fitting Tygon PVC tubing (1/8 in. internal diameter) with nylon barbed plug fittings. PEGDA and HMPP handling was done inside a chemical fume hood because the chemicals are both somewhat volatile.

For crosslinking and photopatterning, a photomask was aligned on the flat bottom face of the device such that it would block UV light from reaching the flow channels and the central culture chamber, while allowing light to reach the cross-channels. The device was exposed to high intensity UV light for 120 s using an Omnicure S2000 spot curing system (Excelitas Technologies, Mississauga, Ontario, Canada) set to 1 W/cm² and fitted with a collimating adapter to make the output intensity approximately 40 mW/cm². Uncross-linked PEGDA was removed from each of the 3 channels as follows. First, PEGDA was removed from the central culture chamber by removing both center channel plugs, withdrawing the fluid using a snugly fitting 1 ml micropipettor and replacing the PEGDA with air, then restoring the plugs. This procedure was repeated with the left channel and then the right channel until PEGDA only remained in the cross-channels, with all other channels filled with air. To ensure all PEGDA was fully cross-linked, the device was once again exposed to UV light using the Omnicure at the same settings as above for 120 s. After this final UV exposure, all channels were filled with ultrapure water.

E. Characterizing transport performance of PEGDA barrier

Performance of the PEGDA barrier to restrict fluid flow and enable diffusion was characterized using dye diffusion experiments and modeling. Toluidine blue was chosen as the tracer due to its favorable optical properties and molecular weight. Toluidine blue can be imaged without fluorescence microscopy and is dark enough at low concentrations to be visible inside smaller microfluidic channels. The toluidine blue molecule (373 g/mol) is similar in size to solutes such as glucose (180 g/mol) and antibiotics such as penicillin (334.4 g/mol), ciprofloxacin (331 g/mol), and ampicillin (349.4) that are commonly used in cell culture studies.

To perform tracer studies, devices were prepared as described above, and then the left and right channels were perfused with either 1 mg/ml (2.67 mM) toluidine blue in deionized water or deionized water at 100 μ l/min using a syringe pump [Fig. 2(a)]. The central culture chamber inlets and outlets remained capped. Time-lapse images of toluidine blue diffusing through the PEGDA barrier were captured on the benchtop using a Dino-Lite Edge 5MP AM7115 Series Digital Microscope (BigC, Torrance, CA) equipped with a diffuser cap; images were taken every 5 min for a total of 4 h. Images were processed using ImageJ (version 1.50i, National Institutes of Health, USA) as described in Sec. II H.

Fluid flow and diffusive transport of tracer dye were modeled in the cell culture platform geometry using COMSOL Multiphysics 5.2 (Burlington, MA) to simulate Fickian diffusion in

FIG. 2. Schematics of the 3D-printed channel device, illustrating where PEGDA barriers were constructed *in situ* in cross-channels connecting the flow and culture channels. (a) Schematic of setup for the toluidine blue tracer dye transport experiment. (b) Schematic of setup for the long-term cell growth experiment. All channels are 2 mm wide and 2 mm high. Device is drawn to scale.

the 3D channel geometry. First, a 3D .STL file of the device's channel geometry was imported into the COMSOL software and a mesh with approximately 30 000 nodes was created for the entire channel interior. Next, laminar flow was defined as 100 $\mu\text{l}/\text{min}$ inlet flow of a dilute solute (left channel) or pure water (right channel), based on the flow conditions in the experimental setup (Fig. S-1, [supplementary material](#)). These flow conditions represent a Reynolds number (Re) of 833 within the specified flow channels, calculated using the microfluidic channel width of 0.2 cm, an average velocity of 0.42 m/s (for a volumetric flow rate of 100 ml/min), and density and dynamic viscosity values for water, since this is a dilute system. This Re value is within the laminar regime for fluid flow in a closed conduit.⁶³ In the simulation, channel outlets were set to gage pressure ($P=0$), with no backflow allowed; the center channel was not allowed any inlet or outlet flow to mimic the plugged chamber in the experiment. The three channels were defined as being filled with water, and a concentration of 2.67 mM at the inlet to the left flow channel and a concentration of 0 at the inlet of the right flow channel, again based on the actual toluidine blue diffusion experiment. The dilute species toluidine blue was assumed to have a diffusivity of $1.42 \times 10^{-5} \text{ cm}^2/\text{s}$ in water and in the hydrogel. In a separate study, we verified the toluidine blue diffusivity computed from the Stokes–Einstein equation of $1.42 \times 10^{-5} \text{ cm}^2/\text{s}$ by matching changes in the dye concentration with position and time in a water-filled microfluidic device with finite element simulations for the same device geometry and experimental conditions.⁶⁴ Lastly, the cross-channels which were filled with PEGDA in the

experiment were defined as separate domains in COMSOL: in separate simulations, this domain was defined as either no-flow with diffusion only (as in the case of an elastic gel fully blocking pressure-driven flow), or where flow was permitted (as in the case of micro-scale pores within the hydrogel permitting convective flow). All liquid-resin or gel-resin interfaces were defined as no-flux boundaries.

F. Bacterial culture preparation

Stationary phase *P. putida* KT2440 cultures were prepared by combining 3 ml of 1× strength 21C medium plus 0.01 mg/ml tetracycline and a freezer stock inoculate in a 15 ml loosely capped culture tube and incubating in an orbital incubator at 30 °C and 120 RPM for approximately 20–24 h. 21C medium was chosen due to its lower autofluorescence compared with complex media such as LB or Tryptic Soy Broth. Cells were washed three times then resuspended (21 000 *g* for 1.5 min) in 1× PBS saline solution. Washed cultures were adjusted to an OD₆₀₀ of ~0.05 (Synergy HT plate reader, BioTek, Winooski, VT).

G. Long-term cell culture experiment

Two identical devices with in-plane PEGDA membranes were prepared as described above, and then pre-treated to minimize biotoxicity. Perfusion channels and the central culture chambers were flushed with ultrapure DI water for 16 h at a flow rate of 50 μ l/min to leach labile resin and photoinitiator from the printed device. Then, inlets and outlets were capped, and the water-filled device was exposed to 30 mW/cm² UV light for 120 s in an ELC-500 UV curing chamber (Electro-Lite Corp., Bethel, CT) to promote further resin cross-linking.

Long-term cell culture experiments were performed as follows [Fig. 2(b)]. First, water was replaced with PBS in all channels using a 1 ml micropipettor. Next, the central culture chamber of one device was loaded with 200 μ l of 0.05 OD₆₀₀ stationary-phase *P. putida* KT2440 suspended in PBS. No cells were added to the central culture chamber of the second device. Finally, both devices were mounted to a glass slide, and placed on an inverted microscope (Zeiss AXIO-observer Z1 with AxioCamMRmRev.3 camera, Carl Zeiss Inc., Germany). Immersion oil was added to the glass slide prior to device mounting to improve clarity; the immersion oil was able to fill in any slight defects or remaining surface roughness of the polished device and improve visibility of the channel interior by minimizing light diffraction from the interfaces. For each device, one channel was perfused at 10 μ l/min with 21C media and the other perfused at 10 μ l/min with PBS *via* a syringe pump in a single-pass configuration for 44 h. Tiled images of the central culture chamber were taken at 2.5× magnification every hour and stitched together using the Zeiss Zen Pro 2.3 software; tiled images of the entire device were taken at 2.5× magnification at 22 h and 44 h and stitched together using Zen Pro.

H. Image analysis for toluidine blue concentration and bacteria growth

For image processing, microscope photos were converted from a color JPEG into an 8-bit greyscale image, where the grey value represented the concentration of dye or bacteria. A higher grey value indicates a darker pixel, while a grey value of 0 is equivalent to a white pixel.

Therefore, in toluidine blue diffusion experiments where the dark blue dye was diffusing into the transparent hydrogel or water-filled channels, a higher grey value indicates a higher toluidine blue concentration. In these images, the grey value of the water-filled channel was measured in five different regions; their area-averaged grey value was assigned a toluidine blue concentration of as 0 mg/ml. Similarly, five images were collected of the toluidine blue flow channel, and their area-averaged mean greyscale value was assigned a toluidine blue concentration of 1 mg/ml (2.67 mM). In the dye diffusion experiments, transects were drawn across the PEGDA barriers in ImageJ at different positions and at different time points. For each transect, the grey value for 0 mg/ml toluidine blue was subtracted from all values, and then all values were divided by the grey value for 1 mg/ml toluidine blue: thereby normalizing transects to a dimensionless concentration from 0 to 1.

In the bacterial growth experiments, a lower grey value (0 = white) indicates an abundance of brightly fluorescing *P. putida* bacteria, while a high grey value indicates a dark channel devoid of bacterial cells. Therefore, in the long-term cell culture experiment, the normalization procedure was reversed. Here, five fluorescent images were collected of the bacteria-free buffer-filled channel, and their area-averaged grey value was defined a bacterial abundance of 0. For the bacterial growth images, transects were drawn through the PEGDA barrier on the media side, across the culture channel, and through the PEGDA barrier on the PBS side [see Fig. 2(b) for device schematic, see Fig. 5(a) for transect example]. This was repeated for each of the three sets of PEGDA barriers for each device at 22 h and at 44 h. Each value in the transect was scaled from 0 to 1 by taking the absolute value of the difference between each measured value and area-averaged grey value measured for a bacterial abundance of 0 (inverting the grey values), and dividing each measured value by the inverted grey value corresponding to the highest biomass observed. In this way, all transects were re-scaled to the same dimensionless scale of 0 to 1.

III. RESULTS AND DISCUSSION

A. Tracer dye diffusion through hydrogel approximated by aqueous diffusivity

Concentration gradients of toluidine blue within PEGDA barriers were visualized experimentally and compared to finite element simulations [Figs. 2(b) and 3]. Flux of solutes through

FIG. 3. (a) Toluidine blue dye diffusing through PEGDA hydrogel barriers, with $100 \mu\text{l}/\text{min}$ flow of toluidine blue in the left channel, deionized water in the right channel, and no flow in the central channel. (b) Time-dependent simulation of toluidine blue concentration, assuming $100 \mu\text{l}/\text{min}$ flow in left and right channels, and no flow in the PEGDA barrier or central channel. (c) Normalized concentration for color-coded transects for experiments (a – symbols) and simulations (b – lines).

PEGDA barriers can also be approximated analytically using unsteady “semi-infinite” 1D Fickian diffusion. Other researchers have used semi-infinite 1D Fickian diffusion assumptions for studying transport through PEG hydrogels.^{33,65,66} The semi-infinite approximation is valid for diffusion into a very wide hydrogel and/or for time points immediately following a step change increase in solute concentration at a boundary (see the [supplementary material](#), Sec. V A and Fig. S-2). For very narrow hydrogels and/or at very long times, the system may also approximate the steady state assumption of a linear concentration gradient in a channel of constant cross-section.⁶⁷ However for irregular geometries and intermediate times, solute diffusion is most accurately modeled using a finite element model such as COMSOL solved for the particular channel geometry and boundary conditions used in the experiment.

In a dye tracer experiment, toluidine blue was observed diffusing through the PEGDA barriers over the course of 4 h. In the images [Fig. 3(a)], flow inlets are at the top and outlets are at the bottom and PEGDA barriers are more opaque white regions in each cross-channel. Over time, the toluidine blue tracer dye advances across the PEGDA barrier until it reaches the central culture chamber between 3 and 4 h. The blue, red, and green data points [Fig. 3(c)] are normalized toluidine blue concentrations along the length of the PEGDA barrier cross-section [Fig. 3(a)]. COMSOL simulation map concentration with space (x, y, and z dimensions) and time [Fig. 3(b)] where black represents the maximum toluidine blue concentration of 2.67 mM and white is a toluidine blue concentration of 0 mM. Simulations assumed unsteady Fickian diffusion of a dilute species with a diffusivity of $1.42 \times 10^{-5} \text{ cm}^2/\text{s}$, the same diffusivity of toluidine blue as in water at 25°C, and no convective flow in the PEGDA-filled cross channels. These simulations were performed on the entire 3D geometry of the device interior.

By inspection, it appears that diffusion occurs uniformly within each of the three barriers, and also occurs uniformly when comparing the first, middle, and last cross-channels (blue, red, and green annotated cross-channels). Comparing the experimental data points and finite element simulations [Fig. 3(c)], the model represents the data fairly well, especially at the initial time points. At time points 120 min and less, the difference between the predicted values and the experimental averages of the normalized toluidine blue concentration at the far boundary of the PEGDA barrier (3000 μm) is less than 10%.

For small solutes, diffusivity in water can often be used to approximate transport in dilute hydrogels. Since the cross-linked matrix is primarily water, molecules smaller than the cross-linked mesh size are able to diffuse through the hydrogel relatively unimpeded according to the free-volume theory of diffusion through hydrogels.⁶⁸ Transport of other smaller molecules frequently used in biological assays, such as sugars, small molecular weight drugs, or oxygen, may also be approximated assuming diffusivities equivalent to those in liquid water. However, charge effects can be important in diffusion through hydrogels, and thermodynamic restrictions including conservation of charge apply.

The concentration at the PEGDA barrier is reasonably well-predicted by the simulations. At 180 min, the difference between experimental averages of toluidine blue normalized concentration and the value predicted by the finite element simulation at the far boundary is 18%. At other positions in the interior of the hydrogel, the difference between the image and the model is somewhat higher. The discrepancy may be caused by the assumption of a linear correlation between absorbance and concentrations or saturation of the optical signal, where it is impossible to differentiate among concentrations that are all very high, or all very low. However, the discrepancy is unlikely to be caused by convective transport through the PEGDA barrier. Additional simulations were performed enabling convective flow, and these results clearly showed concentration differences among the three cross-channels (Fig. S-3, [supplementary material](#)). Our results showed no evidence of systematic concentration differences within or among the PEGDA barriers; therefore, we conclude that convective flow through the barriers did not occur.

The delivery rate of the desired molecule through the hydrogel barrier was the parameter of most interest for bacterial culture applications. Therefore, the delivery rate, or mass flow rate of toluidine blue across the hydrogel barrier was calculated. This value was calculated by determining the flux across the hydrogel boundary into the cell culture channel and multiplying by

the surface area of the hydrogel interface with the channel. Flux was calculated at various time points according to the following equation:

$$J = \frac{D\Delta c}{L},$$

where J is the flux, D is the diffusivity, Δc is the average toluidine concentration at the left boundary of the hydrogel minus the average concentration at the right boundary, and L is the length of the hydrogel barrier. Toluidine blue delivery across the hydrogel barriers and into the central channel was consistent between experimental and simulated values, varying less than 20% between the two for all time points (Table S-1, [supplementary material](#)); at 3 h (180 min) when the system approached the steady state, the calculated experimental toluidine delivery (total sum for all three hydrogel channels) to the central channel was 9.4 $\mu\text{g/h}$, compared the simulated value of 10.3 $\mu\text{g/h}$, only a 10% difference. Therefore, we conclude that finite element simulations assuming no flow in the hydrogel barriers, laminar flow in the left and right flow channels, and Fickian diffusive transport effectively model delivery of toluidine blue tracer dye to the central culture channel.

B. PEGDA barriers impede fluid flow

When comparing the simulation and experimental results side-by-side, a slight difference can be observed, such that the concentration gradient along the hydrogel-filled channel exhibits a sinusoidal shape, while the simulation becomes near linear at 30 min and later. We investigated whether the differences between measured and simulated gradients may have been caused by facilitated diffusion, or a very small amount of flow through the PEGDA barrier. Finite element simulations were performed assuming the presence of flow in the cross-channels. The results of these simulations (Fig. S-3, [supplementary material](#)) show very rapid arrival of dye into the central culture chamber, and uneven dye concentration in the first, middle, and last barrier regions. In contrast, the tracer experiment shows slowly increasing dye concentrations in the hydrogel at 30 and 60 min, and no clear difference in dye concentrations among the first, middle, and last channels.

Finite element modeling simulations where no flow was allowed in the PEGDA barrier region match experimental data better than simulations allowing flow. Therefore, while it is possible that a small amount of flow may occur within the PEGDA barrier, simulations suggest that this flow is negligible, as simulations without flow in the barriers were able to effectively model experimental toluidine blue delivery into the central channel (Table S-1, [supplementary material](#)).

C. Bacteria successfully cultured in 3D-printed devices

The 3D-printed cell culture platform allowed cellular responses to be quantified in real time. Fluorescence signals from the central culture chamber of a device loaded with GFP-producing *P. putida* KT2440 were imaged over 44 h and compared with the signal from a control device where no bacteria were added (Fig. 4). In the images, individual fluorescent objects in the device loaded with bacteria can be easily resolved [Fig. 4(b)]. Comparison with the control device indicates that fluorescence in the central culture chamber is not being caused by the culture medium or PBS saline solution, but by the growing bacteria. Importantly, the PEGDA barriers can also be observed in all images, as they autofluoresce slightly more than the liquid regions, while the SLA printer resin has the greatest autofluorescence enabling clear differentiation between liquid and solid regions.

Bacteria remain viable over the 44 h experiment. The fluorescence intensity of the cells near the left PEGDA barrier boundaries increases between 22 and 44 h, indicating cells are migrating to and growing within this region. Dead bacteria would be uniformly distributed in the central culture chamber. Further, the half-life of GFP is approximately 24 h,⁶⁹ suggesting cells are healthy enough to continue expressing GFP between 22 and 44 h. In transects of

digitally processed images (Fig. 5), normalized fluorescence intensities increase in the device inoculated with *P. putida* from 22 h to 44 h, but not in control devices, confirming cell growth and GFP production over time.

The *P. putida* KT2440 bacteria used in this application is able to withstand exposure to carbon compounds that may be toxic to more sensitive cells in bioremediation applications,^{22,23} so may have been more resistant to leaching resin than other cells may be. Nonetheless, the steps taken to thoroughly rinse the device and expose it to additional UV light prior to adding bacteria may mitigate resin biotoxicity for a variety of other cell types as well, as they did in previous studies with zebrafish embryos.^{18–20}

D. Real-time motility response to chemical gradients

In the microbial culture experiment, stationary phase *P. putida* KT2440 loaded into the central culture chamber was exposed to a nutrient gradient of 21C medium [Fig. 2(b)]. Both media and saline solutions were perfused at a flow rate of 10 $\mu\text{l}/\text{min}$, which resulted in a calculated residence time of approximately 6 min in the perfusion channel. The continuous flow of

FIG. 4. Micrographs of central culture channels with and without GFP-expressing *P. putida* KT2440 over time. The PEGDA hydrogel barriers can be observed as a slightly brighter section in the cross channels, darker than liquid-filled channels. The SLA resin exhibits substantial autofluorescence. The white arrow indicates high concentrations of *P. putida* KT2440 at the PEGDA boundaries where substrates and oxygen concentrations are the highest. (a) Individual images at 25 $\times$ total magnification with 470 nm excitation (GFP emission at 509 nm) and 1000 ms exposure time. Tiles aligned in the Zen processing software. Scale bar 2 mm. The white boxes indicate the approximate location of the detail images shown in (b).

PBS provided the driving force for diffusion, and removed cell waste products. Both solutions were slightly aerated.

Locations of higher fluorescence intensity, and therefore higher bacteria cell concentration, can be observed at the PEGDA barrier boundaries on the side of the media perfusion channel, as indicated by the white arrow [Fig. 4(a)]. This is the location where the substrate concentration in the central culture chamber is the highest, and fresh oxygen is also available by diffusion through the barrier (the hard plastic SLA printer resin has a lower oxygen permeability than the PEGDA hydrogel barrier). Motile bacteria likely congregated at this location because fresh substrate and oxygen were constantly being supplied at the PEGDA boundary. A dimmer band can also be observed on the opposite (right) side of the central culture chamber. Here, aerobic bacteria may also have congregated to access oxygen coming from the PBS. The cell signal is dimmer on the right than on the left because the substrate concentration is not as high as on the media perfusion channel side.

E. Hydrogel effectively excludes motile bacteria

Spatial distribution of fluorescence was quantified along transects using ImageJ (Fig. 5). Greyscale images and transects show abruptly increasing cell concentrations just past the hydrogel barrier. Cells localized at the PEGDA boundary where media and oxygen was perfused. Their abundance decreases with distance from the boundary, and increases again at the far boundary. However, the magnitude of the fluorescence signal along the transect confirms that bacteria are not infiltrating the PEGDA hydrogel. The barrier is able to successfully limit motility and keep cells within the center channel.

IV. CONCLUSIONS AND EXTENSIONS

In this work, a 3D-printed cell culture platform is described where PEGDA hydrogel barriers are fabricated *in situ*. Bacteria cells are cultured in a device comprised of a SLA 3D printed resin for an extended period of time. With steps taken to rinse labile compounds and fully cross-link the resin, bacteria were able to survive and grow over the course of 44 h. To the authors' knowledge, a long-term bacterial cell culture has not been demonstrated previously in a device made of this material.

This platform was used evaluate real-time spatial reorganization of a motile bacterial population in response to differential substrate supply. Cells were observed congregating just outside PEGDA boundaries where substrate and fresh oxygen were delivered to the culture chamber, suggesting hydrogels were able to successfully transport the molecules of interest to cells, while effectively excluding the bacteria themselves. With care taken in device design (minimizing bottom wall thickness), post-production polishing, and mounting with immersion oil, individual fluorescent objects could be resolved at a relatively low magnification. Continued improvement

FIG. 5. (a) Tiled images of 3D-printed cell culture channels converted to greyscale in ImageJ. (b) Normalized fluorescence intensity of the transect shown in (a). The black arrows indicate the PEGDA hydrogel barrier boundaries.

FIG. 6. Schematic illustrating the advantages of thinner hydrogel barriers with fewer posts leading to faster response time and greater diffusive flux, at the potential cost of reduced mechanical stability.

in the optical properties of resin and imaging within devices will further promote its biological application.

We also provide 3D finite element modeling simulations, and show effective diffusivity of the tracer dye was well-approximated using the aqueous phase diffusivity. Although counter-intuitive to some, the hydrogel matrix is still primarily water and the pore size between cross-linked polymers is large enough that the toluidine blue molecules are not hindered. The COMSOL model provided ([supplementary material](#)) can enable researchers in predicting delivery of other small molecules through similarly constructed PEGDA barriers. Methods for characterizing the transport properties of this PEGDA barrier could be repeated with other tracer dye molecules, or molecules with different charge properties, to better understand mass transport of other compounds. Hydrogels with different cross-link densities could be tested to develop barriers with specially tuned delivery capabilities. Ultimately, decreasing response time and increasing flux will be achieved by maximizing the cross-sectional area and minimizing the barrier thickness (Fig. 6). Additional work can be done to investigate and further characterize the interactions between the PEGDA and 3D-printed resin, and surface-modified PEGDA could be incorporated to increase tolerance to pressure gradients and to ensure bacteria are excluded from even narrower hydrogel membranes.

Development of microfluidic technologies has been led by engineering groups proficient with photolithography and soft lithography methods. Highly technical fabrication techniques have created a barrier to broader implementation of microfluidics by biologists.⁷⁰ 3D-printing technology has improved greatly in terms of both performance and affordability in recent years. Therefore, additive manufacturing may provide a point-of-entry for many biologists into micro- and millifluidics, and the simple-to-fabricate cell culture platform described in this paper may be applied by biological researchers to study a wide variety of cell types.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for a detailed explanation of governing equations, assumptions, and initial/boundary conditions for COMSOL Multiphysics finite element simulations. Also included is an explanation of the 1D-Fickian diffusion approximation and a supplemental figure showing the fit of this line with experimental and simulated toluidine blue diffusion. An additional supplemental figure illustrates the simulated toluidine blue transport if convective flow was unimpeded by hydrogel membranes. Lastly, a supplemental table lists calculated toluidine blue delivery values in $\mu\text{g/h}$ for experimental and simulated devices.

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