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Controlled dissolution of freeform 3D printed carbohydrate glass scaffolds in hydrogels using a hydrophobic spray coating

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Abstract

Freeform 3D printing combined with sacrificial molding promises to lead advances in production of highly complex tubular systems for biomedical applications. Here we leverage a purpose-built isomalt 3D printer to generate complex channel geometries in hydrogels which would be inaccessible with other techniques. To control the dissolution of the scaffold, we propose an enabling technology consisting of an automated nebulizer coating system which applies octadecane to isomalt scaffolds. Octadecane, a saturated hydrocarbon, protects the rigid mold from dissolution and provides ample time for gels to set around the sacrificial structure. With a simplified model of the nebulizer system, the robotic motion was optimized for uniform coating. Using a combination of stimulated Raman scattering (SRS) microscopy and X-ray computed tomography, the coating was characterized to assess surface roughness and consistency. Colorimetric measurements of dissolution rates allowed optimization of sprayer parameters, yielding a decrease in dissolution rates by at least 4 orders of magnitude. High fidelity channels are ensured by surfactant treatment of the coating, which prevents bubbles from clinging to the surface. Spontaneous Raman scattering microspectroscopy and white light microscopy indicate cleared channels are free of octadecane following gentle flushing. The capabilities of the workflow are highlighted with several complex channel architectures including helices, blind channels, and multiple independent channels within polyacrylamide hydrogels of varying stiffnesses.

Graphical Abstract

Keywords

direct-write printing; carbohydrate glass; monolithic hydrogel casting; nebulized protective coatings; octadecane; isomalt

1. Introduction

Several bioprinting approaches, including stereolithography (SLA), inkjet, laser assisted (LAB), and extrusion bioprinting are adapted from mature additive manufacturing technologies. [1,2] All of these techniques, however, have a major trade-off between size and printing resolution, [1] requiring larger, high-resolution cell-laden constructs to remain at non-physiological conditions for long periods of time. [3] Moreover, these types of printing require unique materials [4,5] and, due to direct printing, apply non-physiological forces to printed cells. [4,5] An alternative to fabricating cell-laden 3D objects layer by layer is to cast them in a single step. Once the hydrogel is formed, a removable mold, or fugitive ink, is released to produce the final shape. [6,7] State of the art techniques use water soluble polymers, [8] temperature dependent biopolymers, [9,10] and carbohydrate glasses [11] as fugitive inks. These materials provide a temporary negative of the desired geometry thus providing a well-defined and readily printed scaffold for biological fabrication. However, polymers can have slow dissolution kinetics and some viability concerns, [12,13] temperature-dependent biopolymers typically have poor mechanical stability and require co-extrusion with structural components, [10] and carbohydrate glasses dissolve rapidly. [11] Though each material can be used and degradation products are typically benign, modulating their removal can be challenging.

Carbohydrate glasses are especially attractive for biomedical applications because of their broad biological compatibility, ability to form complex shapes, amenability to imaging techniques post clearing, and their rapid removal due to excellent aqueous solubility. Structural rigidity, however, must be maintained while the aqueous gel is setting in order to accurately mold the printed structure in the gel. Miller et al were able to produce tubular networks in various hydrogel systems using log pile arrays of carbohydrate glass by coating them with poly(d,l-lactide-co-glycolide), a biodegradable polymer. [11] While effective, the focus on cell growth overshadowed the characterization of their dip coating procedure, which may not translate to more complex, biomimetic structures. For example, Gelber et al extended the capabilities of sugar alcohol printing by combining the material properties of carbohydrate glass with a purpose built 3D isomalt printer that enabled the generation of

intricate free-form structures. [14] Monolithic casting in polymers showed promise for microfluidic applications including as a dilution generator and a helical mixer, [15,16] but such complex molds have not yet been translated into hydrogels. Approaches such as dip coating or airbrush applications of coatings are enticing due to the ease of use, but manual control limits reproducibility and requires the development of user expertise. [17] Polymeric materials are one coating option to protect scaffolds against dissolution, but can display shape or temperature-dependent degradation kinetics, high material stiffness, and concerns over cellular interaction. [18] In workflows where coatings are left intact, the protective layer could also act as a barrier to cell mobility and transport of signaling molecules or other chemicals, yielding undesirable substrate effects. Control over coating thickness is therefore a paramount concern.

Here we demonstrate a simple method for coating complex, 3D printed isomalt structures for controlling their dissolution in aqueous gels. The validation of a practical approach requires: (a) the choice of appropriate material(s) for coating, (b) providing a practical means to depositing the material, (c) assuring its deposition as desired and, (d) monitoring efficacy of the approach in controlling dissolution. First, we propose to use octadecane (C18) as the barrier material due to its hydrophobicity and low melting point, $\sim 27^{\circ}\text{C}$. [19] Instead of covalent degradation, as required for polymer coatings, octadecane is easily removed with a solid-to-liquid phase transition. The miniscule solubility in aqueous solutions [20] facilitates an effectively physical separation by flushing solution through channels, leaving no detectable C18 after clearing. Second, the challenge in applying any coating to complex parts lies in handling the material and depositing it evenly on the structure. Sprayer systems have been used for a variety of materials and provide access to uniform coatings that could potentially be used to protect sacrificial materials. [21,22] Here we propose and construct an oscillating sprayer system that is optimized by numerical simulation. Third, we design example scaffolds in which our approach can be validated and observe the dissolution process via colorimetry. Finally, we apply a multimodal approach to assess coating efficacy. Contrast-enhanced x-ray computed tomography was used to visualize the entire structure rapidly and in 3D. [23] We used Stimulated Raman Scattering (SRS) microscopy to simultaneously measure the physical and chemical properties of coatings. [24] Both techniques provide exceptional and unique information to optimize the proposed approach, but full clearance of the coatings from the molds is routinely monitored using white light microscopy. Taken together, we develop and validate methods to apply a coating of octadecane which can be fully removed after casting, leaving only the native gel walls.

2. Materials and Methods

2.1 Chemicals

GalenIQ 990 isomalt was a generous donation from Beneo-Palatinit GmbH (Mannheim, Germany). Rhodamine B, octadecane, 1-iodo octadecane, barium sulfate, ethyl alcohol, cholera toxin, insulin, hydrocortizone, and EGF were purchased from Sigma-Aldrich (St. Louis, MO.). Hexanes, donor horse serum, penicillin/streptomycin, phosphate buffered saline (PBS), and DMEM/F12 were purchased from Fisher Scientific (Pittsburgh, PA). Tetramethylethylenediamine (TEMED), ammonium persulfate (APS), 40% acrylamide

solution, and 2% bis-acrylamide solution were purchased from Bio-Rad Laboratories (Hercules, CA). Solid soda lime glass microspheres with median diameter of 7 μm were purchased from Cospheric LLC (Santa Barbara, CA). Substrates were 3D printed using a Form 2 SLA printer from Form Labs Inc using FLGPCL04 clear resin (Somerville, MA). MCF10A cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured following standard protocols. CellTiter 96 proliferation assay was purchased from Promega Corp. (Madison, WI).

2.2 Biocompatibility of mold components

MCF10A cells were cultured to passage number six in DMEM/F12 supplemented with 5% horse serum, 1% pen/strep, 20 ng/mL EGF, 0.5 $\mu\text{g/mL}$ hydrocortisone, 100 ng/mL cholera toxin, and 10 $\mu\text{g/mL}$ insulin following standard protocols. [25] Biocompatibility was determined using a CellTiter 96 well plate proliferation assay following the manufacturer's standard protocol. Briefly, MCF10A cells were plated in a 96 well plate at 5000 cells/well in 100 μL complete medium and allowed to attach for 12h. Cells were then washed three times in 1x PBS and covered in phenol red free complete medium with either isomalt or octadecane at the appropriate final concentration. Phenol red free complete medium was used as a vector for isomalt, while ethanol was used as a vector for C18. After 24h of incubation, cells were treated with 20 μL CellTiter 96 MTS reagent and incubated for 1 hour. Treatment medium was aspirated and replaced on C18 samples with 100 μL fresh phenol red free medium prior to incubation with MTS reagent as the C18 had precipitated from solution and caused scattering artefacts. Absorbance was measured at 490 nm using a Safire II plate reader (Tecan Trading AG, Switzerland). Absorbance values were background subtracted to wells containing phenol red free medium and MTS reagent and normalized to the average control absorbance.

2.3 Freeform isomalt 3D printing

Freestanding sacrificial molds were printed using a custom built 3D printer which extrudes the sugar alcohol isomalt, as previously described (Figure 1A). [14] GalenIQ 990, a 1:1 mixture of glucopyranosido-D-sorbitol and glucopyranosido-D-mannitol, was previously chosen due to its material properties and optimized for extrusion with the custom 3D printer. Two isomalt formulations were used throughout the studies: neat isomalt with a 50 μm inner diameter (ID) nozzle and isomalt dyed with 0.1% (w/w) rhodamine B extruded through a 100 μm nozzle. The operating parameters during printing of each ink were optimized for reproducibility, minimal clogging, and stable beam diameters. Molds were designed in AutoCAD 2018 (Autodesk) to emulate simple luminal structures and other complex systems. Structural beam diameters ranged from 80 to 220 μm while vertical support beam diameters varied between 200 to 400 μm . Variance in beam diameter reflect batch variations between inks; within day reproducibility is within 5%. Typical print times ranged from 0.5–5 minutes per scaffold.

2.4 SLA 3D printing of substrates

Substrates were designed using SolidWorks 2017 (Dassault Systems) and 3D printed using a Form 2 SLA printer to facilitate mold printing, mold coating, casting gels, and clearing gels. Solid nozzles were utilized for printing to improve adhesion of molten isomalt. During

coating, the nozzle offset increases the available arc angle to increase coverage. Finally, a larger initial channel diameter assists with clearing channels. Two tips were incorporated for each through pass channel, while a single tip was incorporated for each terminal channel. Overall substrate height and diameter were optimized to reproducibly position molds for use with Nunc Lab-Tek 16 well chamber slides (Fisher Scientific; Pittsburgh, PA) which have similar dimensions to a 96-well plate. Molds were designed such that horizontal areas of interest were within 500 μm of the slide surface for optical microscopy and Stimulated Raman Spectroscopy.

2.5 Sprayer and Coating

Sacrificial molds were coated with a 100 mg/mL solution of octadecane in hexanes. Coating was achieved using a capillary nebulizer system built in house, as shown in Figure 1. Two degrees of rotation are provided by a stepper motor and a DC motor (Figure 1B and C). Parts to be coated are attached to the DC motor axle with an adapter designed for securely mounting each print. The DC motor operates at a constant rate (960 RPM) during application while the stepper motor oscillates the nebulizing spray head over a 120° arc. The sweep angle was chosen to prevent substrates or the DC motor from blocking the spray path. The center of the oscillation, sprayer-to-part distance, nebulizing gas pressure, solution flow rate, number of oscillations, and sweep velocity are all independent parameters. Here, the sprayer-to-part distance was fixed at 35 mm, compressed air at 40 psi was utilized as the nebulizing gas, and octadecane solution was supplied at 100 $\mu\text{L}/\text{min}$. The oscillation center and sweep velocity were optimized as described in section 2.6. By convention during coating, $z = 0$ corresponds to the substrate tip and the center of the oscillation arc is denoted by C , e.g. $C = 0$ mm indicates the oscillations are centered on the substrate tips. The stepper motor was programmed with a microcontroller attached to a stepper motor controller. The microcontroller triggers motor steps with 5 V pulses delivered to the motor controller. After performing a set number of steps corresponding to 120°, the direction is reversed. Once the desired number of oscillations is complete, the stepper motor stops and the spray is blocked with a glass microscope slide. In order to minimize exposure to the solvent, the sprayer was housed in a fume hood and maintained at room temperature ($\sim 25^\circ\text{C}$). Due to expansion of compressed air from the nebulizer and evaporation of solvent, the area of impact was likely cooled slightly. After coating, sacrificial molds were stored at room temperature in a nitrogen purged environment until casting.

2.6 Sprayer Simulation

Coating uniformity as a function of the oscillation motion was optimized using numerical modeling in MATLAB R2017a (MathWorks). Simulations were performed in the frame of reference of the target object, which was a triangulated sphere mesh of radius 1 with 1026 vertices located at the coordinate origin. Discrete time steps of 1 ms were used for a total of 10 oscillations of the sprayer. The source position rotated at a constant rate of 960 RPM around the z axis of the sphere while the azimuth changed in 0.45° increments, matching 4 microstep divisions of the stepper motor. In the actual sprayer system, the control board operates applying a 1 ms pulse to produce a step. The control pin is then set low for an integer number of milliseconds before supplying the next pulse. The current step position is also recorded. As such, the oscillation velocity is best considered as a series of delay times

determined as a function of step position. While any function is suitable, polynomials are considered here as they demonstrated the best uniformity in initial tests. Specifically, the delay time, d , in ms for the i^{th} step is given by:

$$d = \left\lceil d_{\max} \left(\frac{i}{267} \right)^n \right\rceil + 1$$

where the max delay, $d_{\max}$, and the polynomial degree, n , are free parameters. The constant 267 is the total number of microsteps required to cover a 120° arc in the actual sprayer system. The ceiling function ensures the minimum 1 ms control board delay will elapse. At each time step of the simulation, the current sprayer position is used to determine which vertices are hit by the spray. For the simple model considered here, the sprayer is a uniform, point source. Any unobstructed vertex is coated the same amount regardless of the angle of contact. Under these assumptions with the sphere located at the origin, a vertex, v , is hit by the sprayer at position p if

$$p \cdot v > 0.$$

Once the sprayer path is determined, the total coating at each vertex is summed and normalized to determine relative coating uniformity of a given delay train.

2.7 Colorimetry

The initial dissolution rate of sacrificial molds was determined by colorimetry using isomalt with Rhodamine B. To optimize the center of the sprayer arc, 3 mm tall horizontal molds were coated with a single sprayer oscillation at different sprayer positions and with either constant ($n = 0$) or quadratic ($n = 2$) delay functions. Samples were submerged in ~4 mL of DI water in a cuvette with constant stirring. Absorption readings at 555 nm were collected every 0.5 s over a two minute period with a Jasco V-670 UV-Vis spectrophotometer (Jasco, Easton, MD). The dissolution kinetics were determined as the slope of the absorbance over time for each mold. The effect of print geometry and number of sprayer oscillations were measured similarly, except 1) C was set at -1 mm, 2) parts were submerged in a solution of 10 mg/ml Kolliphor 188, and 3) absorption readings were acquired for up to 10 minutes. The addition of Kolliphor 188 prevented bubbles from clinging to more complex structures.

2.8 Casting and Clearing

In preparation for casting in hydrogels, Kolliphor 188 was applied as a secondary treatment to the octadecane-coated molds to decrease the surface tension of the structure and decrease bubble artifacts in the cast gels. Molds were dip coated for 1 minute in 10 mg/ml Kolliphor in DI water and allowed to dry at room temperature and humidity, typically over the course of 15 minutes. Molds were cast in polyacrylamide hydrogels of various stiffnesses, prepared following previously reported protocols. [26–28] Different stiffnesses were generated by diluting acrylamide and bis-acrylamide solutions to the appropriate percentages with de-ionized water (Table S1). Acrylamide solutions were degassed in vacuum at room temperature for 10 minutes. Cross linking was initiated by addition of 0.5% (v/v) TEMED

and 0.5% (v/v) APS to the degassed solution. Gels were left for >30 minutes at room temperature to allow complete polymerization around the sacrificial molds.

Following casting, molds were cleared by immersing the gels in 37°C de-ionized water. Within 5 minutes, the octadecane coating melts to allow dissolution of the mold, after which printing platforms are removed. Combined with complete immersion, removing the printed platforms greatly enhances separation of the less dense, hydrophobic octadecane which floats to the surface of the water bath. Most octadecane on the exterior of the gels was removed by agitating each well three times with 1 mL of warm deionized water from a transfer pipette. Channels were cleared of residual octadecane by gently flushing 1 mL of warm deionized water directly into the inlets. Terminal channels were more difficult to clear. Instead of relying on flow, pressure was applied using a transfer pipette to gently increase the channel diameter and release trapped octadecane.

2.9 Imaging

Coated isomalt structures and tubes were imaged using a Rigaku GX130 CT Lab x-ray system (Rigaku, The Woodlands, TX). Isomalt scaffolds were imaged in air at 30 kV x-ray energy using a 14 minute, high resolution scan in groups of four scaffolds. For hydrogels, 25% (w/v) barium sulfate or soda lime glass microspheres were used as contrast agents with x-ray energy of 50 kV, 56 minute, high resolution scan with a 25 mm field of view. Glass microspheres were better able to fill blind channels than barium sulfate. Individual parts were cropped out of the scan at 12.5 μm voxel sizes. Coating asymmetry was empirically validated using 1-iodo octadecane at equal molar ratios to the octadecane coating for enhanced x-ray contrast and imaged at 50 kV.

Raman spectra were collected for the isomalt, Kolliphor P188, and octadecane using a Horiba LabRAM HR confocal Raman microscope (Horiba Scientific, Edison, NJ). Octadecane tube surfaces coated with variable numbers of oscillations were imaged for thickness and surface roughness using a home built Stimulated Raman Scattering microscope, [16,29] which allowed for label free, high resolution chemical imaging of the cast structures. SRS images were collected at 2847 cm^{-1} , the CH_2 symmetric stretching vibration, to identify octadecane due to its strong and unique Raman cross section. Scaffolds were cast in polyacrylamide gels for stability and to reduce the number of index of refraction mismatches during measurement. Volumes were acquired at 1 μm^3 voxel size with a width and depth that covered a hemisphere of the tube. Background correction, normalization, and stitching were performed in Matlab as previously described. [29] Thickness was measured from the rotation-corrected Y projection of each tube over a 9 μm width centered on the top of the tube to minimize errors due to scattering artifacts.

3. Results and Discussion

3.1 Oscillating sprayer system design and optimization

The nebulization system, shown in Figure 1, was designed to improve uniformity and consistency of coating compared to hand-held pump or airbrush based systems. Under robotic control, the sprayer traces identical paths between batches and uses consistent gas

and solution flow rates to create more reproducible coatings, as previously described. [17,21,22] As an added benefit, the smaller spray profile decreases the amount of solution consumed during application compared to an artistic airbrush. Typical operating conditions used ~100 μL of coating solution, compared to several milliliters required for airbrush applications. This characteristic becomes significant when more expensive or rare components are applied via nebulization. While several independent parameters of the spray system affect coating thickness, crystal morphology, and droplet size, coating uniformity is a property of the system itself. To a first approximation, the amount of time the sprayer is directed towards a face will determine the coating thickness of that area. To achieve a uniform deposition on 3D surfaces, the sprayer should spend equal time in all positions. However, since the back of the object is occluded by the printing platform, the nebulizer can only cover 2/3 of the sweep path ideally required. One strategy to compensate the angular restriction is to increase the amount of time the nebulizer spends near the end of its travel by dynamically adjusting the oscillating velocity. As another concern, the position of the center of oscillation was found to greatly affect the residence time of molds in water. Thus, the development of a practical approach to spray coat an object needs a careful analysis of the relative motion of the sprayer and object.

To optimize the velocity profile, a simplified system was simulated to track the relative coverage as a function of modulated delay times, as summarized in Figure S1. The simulation tracks spray exposure on a triangulated sphere mesh. Each simulation consisted of 10 oscillations of the sprayer as it rotates around the target; a single downward oscillation at decreased DC rotation is shown in Figure S1A. After determining the exposure for each vertex, as shown in Figures S1C and E, the uniformity is determined as the ratio of the minimum coverage to maximum coverage (Figures S1D and F). The uniformity is bounded between 0 and 1, with 0 indicating the presence of a vertex receiving no coating and 1 when the coating is perfectly uniform. With this setup, arbitrary paths may be evaluated for their resulting uniformity but here, paths were designed with consideration of the stepper motor controller. At each step, the controller delays some integer number of milliseconds before triggering the next step. The initial design choice to use constant delays during oscillations simplifies control but causes non-uniformity on the bottom of the sphere. Due to the incomplete sweep, the bottom receives less exposure and, for any delay time, the uniformity is 0.33 (Figure S1, B-D). In reality, impacts nearly orthogonal to the target will result in less deposition, which lead to even poorer uniformity. To increase coating of the bottom surface, the nozzle may be slowed near the limit of 120° . While several functions were considered during initial optimizations, polynomial relations were found to perform well. Figure S1 B shows a two factor optimization of degree of polynomial and maximum delay time. For short maximum delays, higher degree polynomials generally produce more uniform coatings as more time is spent near the bottom of the sphere. As the maximum delay and polynomial degree increase, the bottom of the sphere becomes more thickly coated than the top. The peak uniformity generally occurs near a quadratic delay function, $n = 2$. While slight variations occur with increased maximum delays, a quadratic delay was chosen due to the ease of implementing the pulse train in the controller. With n set at 2, the maximum delay time was optimized as 35 ms for a total oscillation period of 7 s.

To verify the choice of velocity profile and optimize the arc position, dissolution kinetics were monitored via colorimetry. Triplicate measurements were performed on 3 mm tall, simple bridges coated with a single oscillation (Figure S2A). Regardless of coating parameters, all coated structures dissolved significantly slower than uncoated structures, though the magnitude of the effect varied with oscillation position. With constant velocity sweeps, the minimum dissolution occurred around $C = 1$ mm, with a sharp optimum location. For each spray location, quadratic velocity ramping produced coatings which slowed dissolution significantly more than constant velocity. Additionally, quadratic velocity ramping is more robust to changes in center location; over a range of nearly 4 mm dissolution rates were identical with this kinetic assay. Qualitative measurements indicated spray positions in the range of $C = \pm 2$ mm resisted dissolution for at least an hour (data not shown).

The effect of oscillation center and sweep velocity were also qualitatively visualized by x-ray CT of stands coated with 1-iodo octadecane as a C18 surrogate. The iodo group enhances x-ray attenuation, making it ideal for studying general trends in deposition, however it also causes the compound to have a different crystal structure from non-halogenated C18, [30] making it non-ideal for testing thickness. As shown in Figure S3, the oscillation center of rotation dictates the overall coverage while altering the sweep velocity affects top/bottom uniformity of the coating. Dissolution is more likely to begin at the sites with thin coatings. As such, increased asymmetry at different positions on the structure leads to different modes of failure. Decreased coating at the substrate tips (Figure S3A and C, $C = 3$ mm) causes dissolution starting near the substrate, frequently resulting in the mold toppling off the substrate. Positioning the spray center below the tips ($C \ll 0$) instead causes thinning of the cross-beam coating, leading to dissolution near the corner of the mold. As the spray is moved into an optimal position between these extremes, the entire structure is coated sufficiently to endure water exposure for up to several hours (Figures S3B and D, 2). In comparing the uniformity of constant and quadratic delay scheduling, the horizontal beam appears more evenly coated with quadratic delays (Figure S3C and D). However, quantitative comparisons are difficult due to the resolution of the images and the crystalline morphology of 1-iodo octadecane.

Next, the number of oscillations was optimized by colorimetry with $C = -1$ mm and quadratic delay scheduling (Figure S2B). In addition to increasing the overall coating thickness, additional sweeps are expected to coat areas which are insufficiently protected with a single sweep. This becomes more relevant with complex scaffold geometries with overlapping beams that block the coating from some angles. While the number of passes does significantly affect dissolution rate for single bridges ($p = 0.002$, one way ANOVA), any number of passes beyond two are identical. The small differences between even 2 and 20 sweeps reflects the diminishing returns of thicker coating and the low sensitivity of the assay to nearly impermeable coatings. With additional horizontal sections in the printed part, the trend is similar to the simple case. Perhaps more telling than the differences in mean dissolution rates is their variance. With a single pass, the large variance of dissolution of the bridge with five horizontal sections indicates complex structures are more sensitive to the starting position or manually blocking the spray, which is poorly controlled in the current system. While 2 passes are statistically identical to additional sweeps, 10 oscillations were

utilized for the remaining experiments to increase repeatability of the coating. The choice was prudent for octadecane due to its ease of removal and low cytotoxicity, but for less inert coatings or when matching scaffold geometry is essential, decreasing the number of sweeps is a facile method to modulate coating thickness.

Based on the quantitative measurements and empirical performance of water residence time, the final procedure utilized a quadratic delay with the oscillation center positioned at $C = -0.75$ mm and 10 oscillations. It should be noted that larger molds will require a more complex apparatus or larger spray profile to fully coat scaffolds, but for the 96-well form factor utilized here the sprayer system performs well. As shown in Figure 2, the coated scaffolds last for several hours without noticeable loss of material.

3.2 Material characterization: deposition, removal, cytotoxicity, and morphology

Octadecane is an extremely hydrophobic material with a phase transition at physiologically relevant temperatures ($\sim 27^{\circ}\text{C}$), making it ideal for the coating and removal application. Since the addition of a dye or contrast agent would alter the crystallinity and melting point of C18, all experiments were performed with neat C18. Changes during phase transition were observed with white light microscopy, as shown in Figure S4. Heating the set gel causes the crystalline C18 (Figure S4A) to melt and bead up via the hydrophobic effect (Figure S4B). Solidification of the liquid was evident by the resulting crystalline structure of the C18 droplets upon cooling (Figure S4C). Spontaneous Raman microspectroscopy was used to confirm the composition of the residual droplets. Spectra of pure compounds (Figure S5A and B) indicate octadecane has a significantly higher Raman scattering intensity, likely due to the crystalline structure of solid C18 and the low optical density of acrylamide and dissolved isomalt. Sacrificial structures were then molded and heated but not cleared, and a line scan was taken to identify the residual material (Figure S5C). The extracted intensity correlates strongly with position along the optical image, as shown in Figures S5D and E. Furthermore, spectra corresponding to the droplets match the spectrum of pure C18. Although some differences in the spectra are notable, they likely reflect changes in the crystal structure of octadecane after phase transition, not the presence of contaminants. [31] Following flushing of the channels (Figure S4D) some residual material remains as small ($\sim 7\text{ }\mu\text{m}$) objects. These are most likely small bubbles nucleating on the surface of the octadecane, but may be environmental debris due to handling under non-sterile conditions. For cell culture applications, debris would ideally be removed with further degassing or sterilization by filtration. Either bubbles or debris decreases the effectiveness of the gels; oxygen changes the stiffness, while contaminants could affect cellular behaviors. [32]

Residual mold material could lead to cellular toxicity, therefore cell viability was assessed with concentrations of isomalt or octadecane across their expected abundances. Without dilution in washing solution, the expected final concentration of isomalt in the hydrogel system is no more than 20 mg/mL, with the bridge structure being ~ 4 mg/mL. Based on the coating time and typical coating efficiency, the maximum octadecane concentration without washing is 100 $\mu\text{g/mL}$. As octadecane is not miscible with aqueous solvents, the value is more more likely to be $< 1\text{ }\mu\text{g/mL}$ when octadecane droplets are absent by optical microscopy. Although some shorter chain n-alkanes have been shown to cause toxicity in

macrophages, [33] octadecane did not show any toxicity in the tested epithelial cells. Even at the upper concentrations, both materials caused minimal loss of viability of as shown in Figure S6. The viability measurements suggest the concentrations resulting from sacrificial mold dissolution should be innocuous to seeded cells. This may be due to the much lower concentrations used in our technique than in prior studies.

SRS microscopy was performed on coatings encased in polyacrylamide hydrogels to evaluate the morphology of neat octadecane coated onto isomalt with micron resolution. Although several parameters can affect the final coating (e.g. sprayer-to-part distance, nebulizing gas pressure, solution flow rate, and solution concentration), adjusting the number of oscillations is a simple and rapid modification. Figure 3 shows projections of 3D SRS scans with increasing numbers of spray oscillations. With a single pass of the sprayer, the surface appears evenly coated, with only a few apparent void spots which would be sites of dissolution due to solution exposure.

More oscillations decreases the quantity of void locations. By sectioning the same dataset along an orthogonal direction, the apparent thickness can be evaluated as a function of sprayer oscillations (Figure S7). As expected, thickness appears to increase with additional oscillations, though the coating thickness could not be evaluated fully as signal attenuation and scattering limits the maximum penetration depth to $\sim 30\text{ }\mu\text{m}$, as seen in Figure S7. A single pass of the sprayer produced a coating roughly $4\text{ }\mu\text{m}$ thick, which increases to $5\text{ }\mu\text{m}$ with a second coat and around $8.5\text{ }\mu\text{m}$ with 5 passes. Note these values are obtained from single cross sections; the projections displayed in Figure S7 represent the maximum extents of the coating including protruding crystalline structures. Although increasing the number of coats decreases the number of voids, the size of crystalline structures also increases. Even with a single pass, crystal structures are visible that span tens of microns. Increases in surface roughness have been shown to change cellular morphology, but changes have been shown in the nano-scale for epithelial and endothelial cells. [34,35] Micro-scale topography has been shown to have less of an effect on epithelial proliferation than fibroblastic proliferation, [36] so in our case the increased barrier function may be worth the increase in crystallinity.

3.3 Evaluation of Molding Fidelity

While the hydrophobicity of octadecane simplifies removal, it also poses problems during casting into aqueous solutions. Due to the high surface tension between octadecane and water, structures with small features or high planar surface areas do not wet properly, in this case regularly trapping bubbles within internal angles (Figure 4C). Surfactants have been shown to decrease the contact angle of solutions and increase solvent spreading. [37] To cast channel geometries without capturing bubbles, a secondary coating with surfactants was applied to decrease the hydrophobic effect of C18-coated structures. Kolliphor P188 was selected as it is commonly used in cell culture to decrease shear forces and has been shown to be non-toxic at up to 10 mg/mL concentrations. [38] Though several methods of applying Kolliphor P188 successfully prevented bubble adhesion, dip coating was selected due to its simplicity and throughput. An added benefit of dip coating is quality control; scaffolds which fail to resist dissolution during dip coating are unsuitable for casting into hydrogels.

As shown in Figure 4D, once the coating dries it effectively eliminates large bubbles from the hydrogel mold. With the two step coating process, channels produced with the sacrificial isomalt constructs closely replicate the desired geometries.

While local structure can impact cell fate, the stiffness of a cell's surroundings controls differentiation potential, morphology, and general cell health. [39,40] As such, the effect of hydrogel stiffness was next investigated. The fidelity of structures produced with soft gels is typically poor in bioprinting applications as the gel is incapable of supporting its own weight, leading to deformation. [41] A particular concern for the mold clearing protocol described in section 2.8 is that soft gels are difficult to manipulate, as they can deform or rupture under application of light pressures, in some cases requiring custom built systems to measure minuscule forces. [42] However, as shown in Figure S8, the three channel structures are accurately produced in polyacrylamide gels spanning the physiological range of stiffness from 0.08 kPa (a factor of two below healthy human mammary gland [43]) to 120 kPa (three fold stiffer than a human breast tumor [44]). No loss of fidelity was observed at low stiffness, which we attribute to the weak adhesion of liquid octadecane to the aqueous gels and the increased cohesion of the monolithic cast. Though soft, the polyacrylamide gel withstands the gentle flushing required to remove the protective coating. The isomalt/octadecane sacrificial mold appears to be agnostic to gel stiffness, the only restriction on matrix composition is that setting must be performed at or below room temperature and the gel must be cohesive enough to survive flushing.

Coupling our unique 3D printer capabilities with the recently developed surface protection, casting complex 3D channel structures in hydrogels is now feasible. Figure 5 displays a few representative parts which become tractable with the developed workflow. Each would be difficult to produce quickly with traditional bioprinting or other carbohydrate glass systems. Figure 5A displays a horizontal helical structure. 3D printing the helix mold required a two-step process so the helix is printed vertically, however this could potentially be done by modifying the 3D printer with additional axes to prevent the nozzle from colliding with previously printed paths. [45] After casting, a small bubble is evident on the right side of the structure, but generally the fidelity is exceptional. Multiple channels are also simple to implement, as shown in Figure 5B. Here two boldface I shapes are produced with an offset to generate independent channels. Again, the final channel fidelity closely matches the fugitive ink mold. While through channels simplify clearing the melted octadecane, blind channels are also feasible without modification. Figure 5C displays a simple branching structure, similar to a breast, alveolar, or hepatic duct. Note in the line-path model the branches are offset by 100 μm to prevent melting of cantilevered beams during printing. Each of these modular ideas may be combined, as shown in Figure 5D, which is a system with 3 independent channels and a blind branch, which could model venous and arterial blood flow around a branching epithelial system. While shadowing of the coating is a concern with any projectile-based coating system, the sparse, direct-write designs the isomalt printer is optimized for do not occlude enough of the spray to prevent deposition of a protective coating. The mold in Figure 5D took less than five minutes to print, whereas recreating a similar level of complexity and resolution with a traditional bioprinter would be time consuming. Moreover, in a traditional bioprinter setting, cells would be confined to the printer during the entire process. Using the molding technique above, the earliest that cells

would be introduced would be during gelation in extracellular matrix hydrogels using previously described protocols. [46]

4. Conclusions

Here we show the combination of sugar alcohol 3D printing, spray application of hydrophobic coatings, and surfactant treatment to produce intricate channels within hydrogels. After casting, the sacrificial mold is cleared through immersion in a water bath and gentle flushing, leaving no detectable coating material, namely octadecane. Through the use of modeling and spectroscopic imaging, the automated nebulizing system is shown to uniformly cover the 3D prints with a crystalline layer. Optimized octadecane coating decreases the rate of isomalt dissolution in water by at least four orders of magnitude. Each component is biocompatible, which is vital for producing biomimetic structures. Furthermore, the nebulizer should be suitable for coating a variety of components including polymer coatings, aqueous solutions and possibly cells. This work is a critical step in providing the flexibility to generally design sacrificial systems that can investigate cellular phenotypes when exposed to diverse channel architectures, gel stiffness, and matrix composition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic of printing and coating. (A) Frame of reference for printing. The nozzle extrudes molten isomalt during direct-write printing. (B) In a second step, the isomalt mold is coated with octadecane in a custom-built nebulization system. The part continuously rotates around the z axis at 960 RPM while the spray nozzle oscillates between 0 and 120°. The tip of the substrate is defined as $z = 0$ and the center of oscillation, C , is optimized for maximum water residence time. (C) A rendering of the sprayer system.

Figure 2. Qualitative dissolution kinetics. Isomalt containing Rhodamine B was printed into 3 mm straight bridges. The left two parts are uncoated and the right two are coated with octadecane using optimized parameters. Uncoated isomalt scaffolds completely dissolve within seconds while the octadecane-coated pieces remain intact in water for several hours.

Figure 3.

Chemical analysis of sacrificial mold materials. (A) Normalized spontaneous Raman spectra of polyacrylamide gel, neat isomalt, octadecane, and residual droplet material showing the frequency chosen for SRS imaging. (B-F) Chemical imaging of coating morphology with increasing number of oscillations. Images are two dimensional maximum Z projections of the three dimensional tubes. Uncoated areas are highlighted with arrows. (B) 1, (C) 2, (D) 5, (E) 10, and (F) 20 oscillations.

Figure 4. Surfactant coating improves hydrogel fidelity. (A) Computer model of desired architecture. Spiral bases are utilized for intimate contact with the substrate tips, shown in blue. (B) Volume rendering of x-ray computed tomography dataset for octadecane-coated isomalt structure. Larger beam diameters were utilized for the vertical channels to assist with clearing. (C) CT volume rendering of channels formed in polyacrylamide gels with octadecane-coated scaffold. (D) CT volume rendering of octadecane-coated scaffold dip-coated with Kolliphor 188 and cast in polyacrylamide hydrogel.

Figure 5.

Examples of complex channel morphologies generated with octadecane-coated, pluronic-treated isomalt structures. For each panel, the design, isomalt structure, and resulting channels are shown from left to right. (A) Helix, (B) two independent channels in the shape of a bold-face I, (C) branching, blind channel, (D) three, independent channels intertwined.