Parylene-C coating protects resin 3D printed devices from materials erosion and prevents cytotoxicity towards primary cells

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ABSTRACT Resin 3D printing is attractive for rapid fabrication of microscale cell culture devices, but common resin materials are unstable and cytotoxic under culture conditions. Strategies such as leaching or over-curing are insufficient to protect sensitive primary cells such as white blood cells. Here, we evaluated the effectiveness of using parylene-C coating of commercially available clear resins to prevent cytotoxic leaching, degradation of microfluidic devices, and absorption of small molecules. We found that parylene-C significantly improved both the cytocompatibility with primary murine white blood cells and the material integrity of prints, while maintaining the favorable optical qualities held by clear resins.

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Resin 3D printing is a popular choice for microfluidic fabrication due to its ability to efficiently create complex devices, and in principle is well suited for iterating and prototyping organ-on-chip and other cell culture devices. However, most photopolymerizable resins used for resin 3D printing are highly cytotoxic,[1–7] especially for use with primary cell cultures such as white blood cells.[8] Furthermore, many of these materials also are limited in reusability because they wear down over time, erode or delaminate (especially at temperatures of ~37°C and greater),[8] and absorb media components into the prints. Many of these problems are due to leaching of unbound compounds such as monomers, surfactants, or photochemical compounds.[9–13] Depending on the material, carefully developed methods for over-curing and/or extraction have increased the compatibility of resin prints with some immortalized cell lines,[11,14] but more fragile primary cell cultures and developing embryos are still vulnerable.[6–8,14] Additionally, we have found that some materials deform or delaminate during extraction.[8] Currently, the twin challenges of cytotoxicity and material instability have relegated many 3D printed devices to non-biological workflows, such as for use as a master mold for rapid prototyping (e.g. of PDMS devices). Solving these problems is critical to enabling direct production of cell culture substrates and microfluidic devices for primary cell-based assays and organs-on-chip by 3D printing.

An alternative to removal of uncrosslinked components is application of an impenetrable barrier between the material and its environment. Indeed, neural or cardiovascular implants and stents are often coated with a polymeric layer of parylene to prevent water absorption and material disruption.[15] Parylene-C is the most affordable and commonly used derivative of the para-xylylene polymers and is conformally applied via vaporization. Once a thin layer is formed over the material by chemical vapor deposition, it creates a barrier that is impermeable to many molecules, including water, and is insoluble in organic solvents below ~150°C.[16] In addition to coating surgical

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materials, parylene-C was used as substrate for microscale cultures in early microfluidics and micropatterning work.[17,18]

Recently, a few reports have shown that coating resin 3D printed materials with parylene-C increased their compatibility with hardy cell lines in direct or indirect contact with the materials.[19–22] For cells cultured directly on the 3D printed substrate, parylene coating has been used successfully with endothelial cells (HUVECs)[22] and the epithelial cell line MDCK.[19] Parylene was also effective in an indirect context, i.e. for culture of cell lines including keratinocyte HaCaTs[19] and primary neuronal cells on PDMS chips molded from parylene-coated 3D printed master molds.[20] While these results were promising, leaching and extended curing were similarly successful with most cell lines and yet of limited efficacy for many primary cells.[6–8,14] Therefore, it was critical to explicitly test the extent to which applying parylene to 3D printed devices would act as a protective layer between sensitive, heterogeneous primary cells and the cytotoxic material, while also potentially increasing material integrity of the device (**Figure 1**).

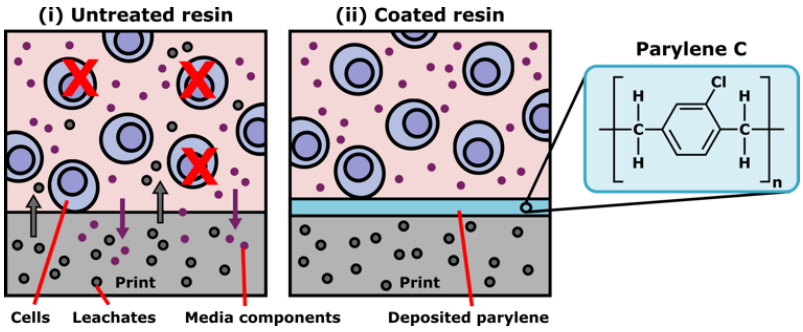

Figure 1. Principle of protection by parylene-C coating. (i) Toxic leachates (grey filled circles) diffuse out of 3D printed materials and interact with cells in culture while causing a loss of material stability. Meanwhile, absorption of small molecules (purple dots) into the prints may deplete media

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components or contribute to material degradation. (ii) Coating with parylene-C (teal) creates a barrier that prevents absorption or leaching to and from the print.

Here, we evaluated this coating for biomicrofluidic device fabrication by testing the cytocompatibility, material properties, and optical properties of coated versus uncoated resin 3D prints of varied geometry. 3D printed wells (**Figure 2a**) and microchannel chips with raised port inlets (**Figure 2b**) [23] were used for the evaluation of cytocompatibility and material integrity, while 8-mm cubes (**Figure 2c**) were used to characterize surface and optical properties. All prints were post-treated by washing with isopropanol and post-curing with UV light (details provided in **Protocol S1**).[8] To coat the prints after post-treatment, parylene-C (Specialty Coating Systems, USA) was deposited in ~1 μm-thick films using a Labcoater 2 (Specialty Coating Systems, USA), as detailed in **Protocol S2**.

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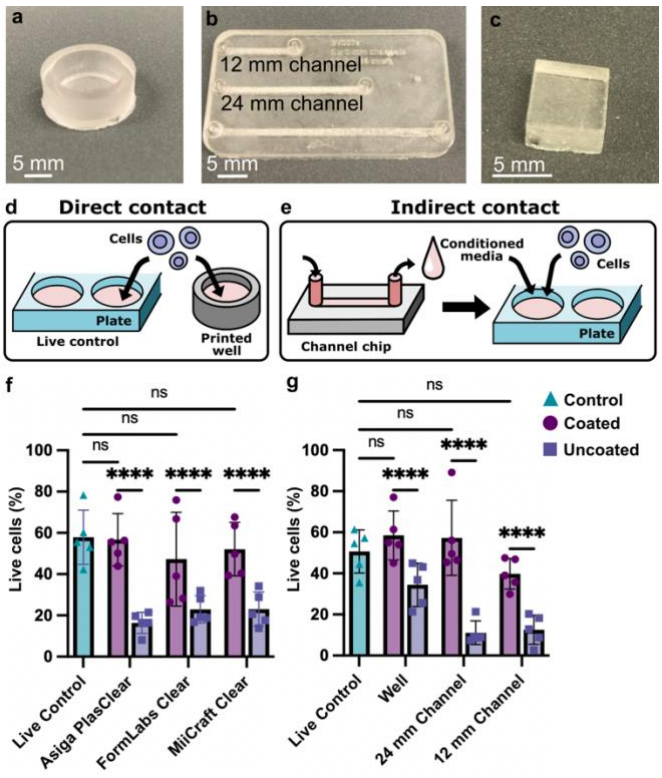

Figure 2. Parylene-C coatings improved cytocompatibility of 3D printed materials. (a-c) Images of 3D printed test pieces: (a) well with 750 µL volume and 1 mm wall thickness, (b) multichannel chip with square, 1x1 mm^2 cross-sectional channels of 12 mm and 24 mm lengths, each with raised ports at the inlets and outlets, and (c) an 8 mm cube. MiiCraft Clear resin is shown. (d-e) Schematics of the two culture setups: (d) direct contact of cells on 3D printed wells, and (e) indirect contact, i.e., cells cultured in conditioned media collected from 3D printed microchannels. (f-g) Viability of primary murine splenocytes after overnight culture in (f) direct contact with different resins or (g) indirect contact with different geometries in MiiCraft Clear prints. Live cells were determined via flow cytometry as the percent of Calcein-AM^{High} and $7AAD^{Low}$ out of total cells. Bars show mean with standard deviation (n = 5). Each dot represents one sample (~0.5 x 10^6 cells/sample) in the listed media condition. Two-way ANOVA. $^{ns}p > 0.08$, $^{****}p < 0.0001$, coated vs uncoated.

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First, we evaluated cytocompatibility with primary murine white blood cells that were cultured directly on the printed materials (**Figure 2d**). We have previously shown that white blood cells are particularly sensitive to toxins,[8,24] making them a rigorous test of cytocompatibility. We selected three resins commonly used for microfluidic fabrication – Asiga PlasClear, FormLabs Clear, and MiiCraft Clear – and used them to print small culture wells (**Figure 2a**). The wells were designed to match the dimensions of a 24-well plate, thus allowing both physical contact between the cells and the bottom of the well, as well as a surface-area-to-volume ratio comparable to traditional cultures. Primary murine splenocytes were collected as previously described (**Protocol S3**) and cultured overnight (24 hr at 37°C, 5% CO_2, 1 x 10^6 cells/mL) within coated and non-coated 3D printed wells, or in a standard non-treated polystyrene 24-well plate used as a "live" control. Flow cytometry was used to evaluate viability, outlined in **Protocol S3** with controls in **Figure S1**. As expected, the uncoated prints resulted in significant toxicity (**Figure 2f**). In contrast, the parylene-C coated prints demonstrated excellent cytocompatibility, with no significant difference in viability from standard well plates. However, variation did occur with the FormLabs clear resin, suggesting further evaluation of surface properties or coating thickness for this material would be beneficial.

While the printed culture well was an open device, many microfluidic devices use internal features, such as interior channels with small inlets and outlets. We next tested the extent to which the vapor deposition process was sufficient to protect internal microchannels, by positioning a channel upstream of cell culture and thus indirectly in contact with cells (**Figure 2e**). All microchannel chips were printed in MiiCraft Clear resin (formerly called Miicraft BV007a). Cell culture media was flowed through the channel using a Fusion 720 syringe pump (Chemyx Inc., USA) at a rate of 2 µL/min overnight (~18 hours) at 37°C, or incubated in wells for the same

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period. As a "live" control, media was flowed directly from the syringe into collection tubes (no chip). Splenocytes were then cultured in these media for 24 hr in a standard polystyrene well plate. As in the direct viability experiment, the media conditioned by uncoated channels resulted in a significant drop in viability, whereas coated wells and channels had no significant impact on cell viability (**Figure 2g**). We concluded that the parylene coating provided a sufficient barrier to keep toxic leachates from entering the flowing media in these channels. Efficacy of coating enclosed features, such as channels, will depend on geometry (length, cross-sectional area) and how long they spend in the coater.

We next tested the effects of parylene coating on material integrity and found that it protected printed microfluidic devices from erosion that can occur during use (**Figure 3a**). We designed a test experiment consisting of sterilization and overnight perfusion with media (2 μL/min) at 37°C, similar to typical biomicrofluidic experiments (**Protocol S4**). Brightfield images showed that internal flow channels printed in MiiCraft Clear resin (**Protocol S5)** became visibly cracked and damaged (**Figure 3a,** "uncoated") after three uses in this experiment. MiiCraft Clear has a high additive content (10-15%) and thus may be prone to erosion as components such as plasticizers leach out of the printed material.[8] The parylene coated channels, in contrast, maintained intact walls after the same number of uses (**Figure 3a**, "coated"). Additionally, we found that in uncoated chips, the printed ports were more likely to fail and leak from wear and breakage than in coated chips. These results suggested that parylene-C is protective against the erosion of resin 3D printed channels and ports, thus allowing for a larger number of re-uses from the device.

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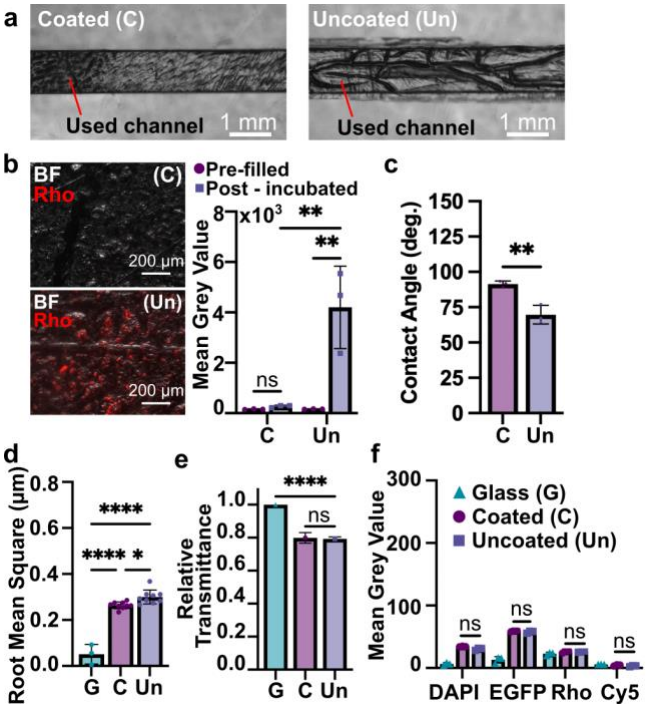

Figure 3. Parylene-C acted as a protective barrier to preserve material integrity without modifying optical properties of the 3D prints. All tests were conducted with MiiCraft Clear resin. (a) Brightfield images of coated and uncoated channels after three uses, where each use included cleaning and overnight perfusion with media (2 µL/min) at 37°C. (b) Overlaid fluorescence and brightfield images (11.2x magnification) of coated and uncoated 3D printed wells after overnight incubation with rhodamine solution. Plot shows quantification of fluorescent intensities before ("pre-filled") and after ("post-incubated") incubation. Two-way ANOVA (n=3). $^{ns}p > 0.9999$, $^{**}p > 0.001$. (c) Contact angle measurements of printed surfaces. Unpaired T-test, $^{**}p = 0.0057$ (n=3). (d) Surface roughness of printed surfaces, with glass (G) control, in root mean square deviation. One-way ANOVA (n=3 measurements per piece with 3 sample pieces per condition and 1 control piece). $^{*}p = 0.0284$, $^{****}p < 0.0001$. (e) Relative transmittance of white light through printed cubes, with glass control. One-way ANOVA (n=3). $^{ns}p = 0.9051$, $^{****}p < 0.0001$. (f) Fluorescent

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intensity of prints, with glass control, in DAPI, EGFP, Rhodamine, and Cy5 channels. Two-way ANOVA (n=3). $^{ns}p > 0.1$. All bars in graphs show mean with standard deviation.

In separate experiments to assess absorption of compounds from the media, a fresh set of coated and uncoated printed wells (not previously used) were incubated overnight (37°C, ~20 hrs) with Rhodamine WT (a water-soluble rhodamine dye, 567 g/mol, 2% w/w in water, Acros Organics, Germany), then rinsed and dried. Parylene coating reduced the absorption of small molecules into the prints by 15-fold, down to baseline levels (**Figure 3b**). This property is expected to enable delivery of precious reagents without loss to the walls and without cross-contamination during sequential treatments.

Consistent with these results, parylene converted the material from moderately hydrophilic (<78° contact angle) to moderately hydrophobic (>90°) (**Figure 3c,** collected with a goniometer, Ramé-Hart Instrument Co., USA; 2 µL droplet of DI water in air) and smoothed out the material slightly as it filled in nanoscale gaps on the surface (**Figure 3d, Protocol S6**). Both surface properties are expected to be adjustable, as the hydrophobicity of parylene is modifiable via oxygen plasma treatment,[25,26] and micro-sanding could be performed prior to coating, if desired.

Finally, although we observed that the parylene-C coatings visibly refract light, neither the transmittance of white light (**Figures 3e, S2**) nor autofluorescence emission (**Figures 3f, S3**) of the material were altered by the coating. These properties were measured according to our reported protocols (briefly in **Protocol S4**).[8] It is likely that optical clarity could be further improved by increasing the smoothness of the 3D prints prior to coating, as much of the light scattering occurs due to its surface roughness.[8,27]

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In conclusion, parylene-C coating of resin 3D printed devices provided protection against cytotoxicity with primary white blood cells regardless of resin used and was effective on open wells and interior microchannels with open inlets and outlets. The coating also substantially increased material integrity by protecting against erosion and molecular absorption and maintained the microscopy-compatible optical properties of clear resin prints. With the compatibility of parylene with organic solvents up to 150 °C,[16] it is also possible that the coating may enable use of 3D printed devices to other chemical applications, although this was not tested here. Due to the sensitivity of primary white blood cells, we anticipate that they are a high bar for testing, and thus that parylene coating of 3D prints may be broadly effective with many cell types, although these should be tested individually. Therefore, this well-established chemical treatment may extend the use of rapidly fabricated and potentially re-usable 3D printed microfluidic devices to on-chip culture of primary cells, organs-on-chip, and other biomicrofluidic applications.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Electronic supplementary materials (PDF), including a link to access 3D printed files used in the main text, Supplemental Figure S1: Gating strategy and single stain controls for flow cytometry, Supplemental Figure S2: Combined transparency data for four commercial resins, uncoated and coated, Supplemental Figure S3: Combined autofluorescence data for four commercial resins, uncoated and coated, and Supplemental Protocols S1 – S6.

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Author Contributions

The manuscript was written through contributions of all authors. Conceptualization: HBM, RRP; Data curation: HBM, SRC, RRP; Formal analysis: HBM, SRC; Funding acquisition: RRP; Investigation: HBM, SRC; Methodology: HBM; Visualization: HBM, SRC; Writing – original draft: HBM; Writing – review & editing: HBM, SRC, RRP. All authors have given approval to the final version of the manuscript.

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