Exploring Endothelial Cell Adhesion to High-Resolution 3D Printing Materials for Advanced Organ-on-Chip Fabrication

Steffen Winkler[1][a], Xenia Kraus[1][b], Jasmin Huber[1] and Janina Bahnemann[1,2][c]
[1]*Institute of Physics, University of Augsburg, 86159, Augsburg, Germany*
[2]*Center for Advanced Analytics and Predictive Sciences, University of Augsburg, 86159, Augsburg, Germany*

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Abstract: With advancements in resolution, 3D printing is emerging as a transformative technology for the rapid fabrication of cell culture systems, including organ-on-chip platforms. For successful integration into cell culture environments, 3D printing materials must not only exhibit general biocompatibility but also support direct cell adhesion for on-chip cultivation. In this study, we investigated the adhesion of human umbilical vein endothelial cells (HUVECs) to two 3D printing materials, AR-M2 and M2S-HT90, under varying sterilization conditions involving heat steam sterilization and ethanol disinfection. Our findings reveal that specific combinations of these sterilization techniques significantly enhance cell adhesion, achieving levels comparable to standard cell culture plates. However, alterations in the 3D printing mode resulted in a complete loss of cell adhesion, underscoring the critical impact of printing parameters on the material surface properties.

1 INTRODUCTION

Organ-on-chip (OOC) systems simulate increasingly complex tissue and organ functions and serve as novel cell culture platforms – enabling both a deeper understanding of (patho)physiological processes in academia and industrial applications such as drug discovery. They are increasingly recognized for their essential role in mimicking the complexity of tissues and organs, thereby enhancing the physiological relevance of experimental results (Leung et al., 2022).

Traditionally, OOCs are microfluidic systems that consist of microchannels and chambers enabling active perfusion of the culture with medium. Fabrication of microfluidic systems remains a considerable challenge. Traditional manufacturing techniques are time- and cost-intensive such as soft lithography, micro-milling, injection molding, and etching, which necessitate the use of highly specialized equipment and cleanroom facilities.

Recent advancements in 3D printing have introduced promising alternatives for fabricating OOCs more cost-effectively and with reduced developmental timelines (Meyer et al., 2023; Siller et al., 2020). Nonetheless, there are only few peer-reviewed studies evaluating the biocompatibility of 3D-printed materials that can be printed in high-resolution (Siller et al., 2019; Winkler et al., 2022). Biocompatibility assessments are typically conducted according to established guidelines, such as the International Organization for Standardization (ISO) norms (e.g., ISO 10993) and United States Pharmacopeia (USP) Class VI standards.

Despite these assessments, the direct growth of cells on 3D-printed materials – specifically the adhesion of cells to the material surface – is rarely investigated, as this is not a mandatory criterion in ISO-based biocompatibility evaluations. However, cell adhesion of a given cell line, is crucial for the development of OOCs. It facilitates the use of cell culture systems that are printed in a single step for immediate on-chip cell seeding. When investigating the adhesion properties of a 3D printing material, material post-processing, including the sterilization method, must be considered, since it can significantly influence the materials surface properties and the amount of potentially toxic leachables.

[a] https://orcid.org/0000-0001-7386-9477
[b] https://orcid.org/0000-0002-1186-5443
[c] https://orcid.org/0000-0002-7008-1673

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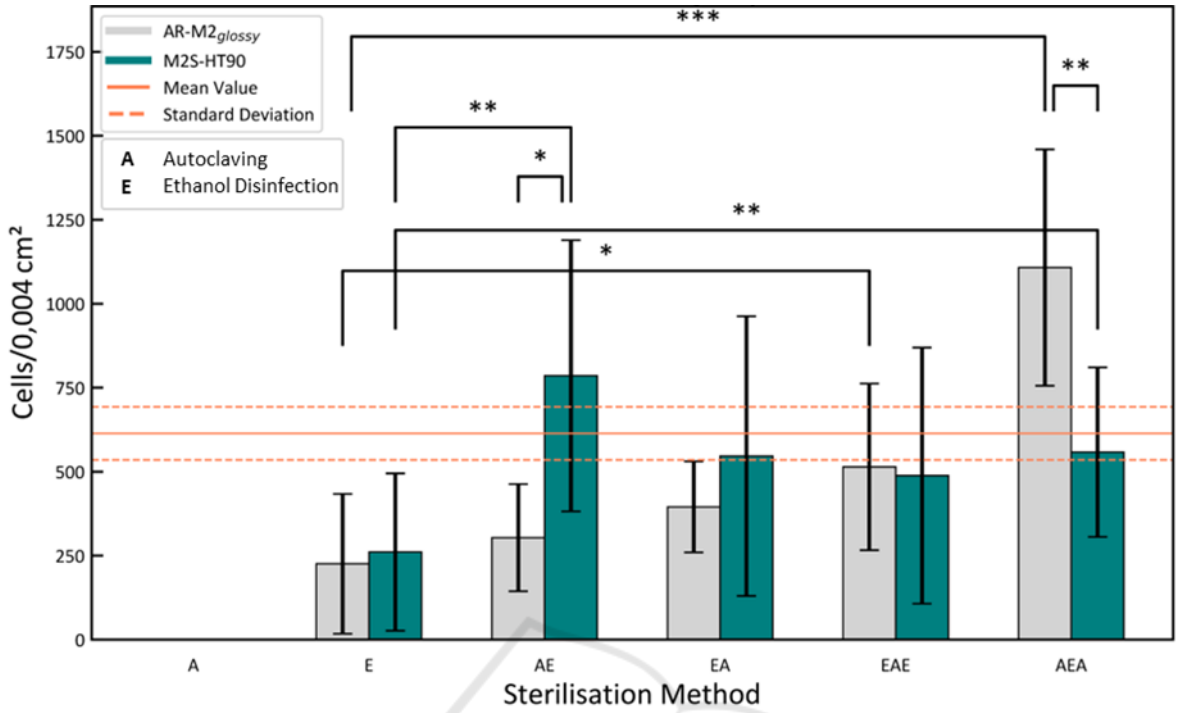

Figure 1: Cell adhesion of human umbilical vein endothelial cells (HUVEC) to material slides of the 3D printing materials AR-M2 (printed in *glossy* mode) and M2S-HT90 for different sterilization/disinfection procedures using autoclaving (A) and/or ethanol (E). Mean and standard deviation of the control is indicated by orange lines. Level of significance as indicated by asterisks, *p < 0.05; **p < 0.01; ***p < 0.001.

In this study, two materials designed for high-resolution 3D printing of microfluidic cell culture systems were evaluated for their ability to support the adhesion of human umbilical vein endothelial cells (HUVEC). The results revealed that different combinations of sterilization methods and printing modes had a pronounced impact on cell adhesion. The findings underscore the importance of material selection, post-processing, and evaluation in advancing the development of functional and reliable OOCs.

2 RESULTS AND DISCUSSION

Two 3D-printing materials, AR-M2 (printed with AGILISTA-3200 W) and M2S-HT90 (printed with ProJet MJP 2500 Plus), were tested for their adhesion properties to HUVEC. Hence, the cells were cultivated on thin 48-well material slides (see Figure S1 in the Appendix), which were sterilized using different combinations of heat steam sterilization (autoclaving, (A)) and ethanol disinfection (E) as two of the most popular sterilization/disinfection techniques. Both materials are described by the manufacturers as biocompatible according to ISO 10993 and/or USP Class VI and can be printed in high resolution for the fabrication of microfluidic structures and OOCs.

The cell confluence (as cell count per imaging area of 0.004 cm²) after 24 h cultivation on AR-M2 (printed in *glossy* mode) and M2S-HT90 material slides for different sterilization procedures is summarized as presented in Figure 1. Noticeably, the confluence increases with an increased number of sterilization/disinfection steps. Slides that were only autoclaved (A) facilitated no cell growth at all, while ethanol disinfection (E) with 225 ± 208 and 261 ± 234 cells/0.004 cm² shows a significantly impaired adhesion compared to the control with 614 ± 78 cells/0.004 cm². For the M2S-HT90 material all combinations of ethanol and autoclaving treatments showed no significant difference to a 48-well plate and thus enable the use of the material for direct on-chip cultivation. Considering these findings, it is hypothesized that potential leachables are increasingly released to the surface of the material due to the heat of the autoclaving process and lead to a fully impaired cell adhesion. In turn, additional extraction by ethanol removes these leachables and a combination of both sterilization methods leads to an overall decrease of leachables inside the material and potential cytotoxicity. However, for the AR-M2$_{glossy}$ highest confluence

was found for the AEA sterilization sequence. Since also ethanol can diffuse into the material, it may not be removed by subsequent washing steps. The heat of a second autoclaving step could reduce a possible negative effect of ethanol and thus further reduces impairments of cell attachment and growth. In addition, representative images of the control and the best performing sterilization/disinfection procedures are presented in Figure S2 showing a similar cell distribution and morphology.

Importantly, the AR-M2 material was also printed in in the *matte* printing mode, where in contrast to the *glossy* printing mode additional support material covers the top surface of the material slide. Surprisingly, the change of the printing mode led to a complete loss of cell adhesion under all tested conditions (data not shown). This is explained by a complete change of the surface structure in the *matte* printing mode, which increases the surface roughness. The findings demonstrate the importance of the printing mode and its dramatic influence on cell adhesion.

3 CONCLUSIONS

In this work, two materials AR-M2 and M2S-HT90 – suitable for high resolution 3D printing of microfluidic and OOC systems – were investigated on their adhesion properties to HUVEC using several different sterilization procedures. The findings show that combinations of the sterilization techniques enable the use of the materials for direct cultivation of HUVEC with a comparable confluence to standard cell culture plates after 24 h cultivation. Furthermore, it revealed that solely sterilizing by autoclaving or disinfecting using ethanol is insufficient and is accompanied by an impaired cell attachment. Another key finding is the strong effect of the printing mode. The *matte* printing mode of the AGILISTA-3200 W results in a loss of cell adhesion at all tested conditions. Since the surface structure in generally highly dependent on a variety of printing parameters, change of these parameters can generally remove or restore cell adhesion of 3D printing materials.

In contrast to few publications focussing solely on the biocompatibility using extraction media according to ISO-10993, we enabled the use of the printing materials for direct cell growth on the inner surface of future cell culture systems. It enables the development of OOC systems that are fabricated as a single part removing the need for the integration of external materials and thus further reducing fabrication and development times.

4 EXPERIMENTAL SECTION

4.1 Fabrication and Post-Processing of 3D-Printed Parts

The 3D-printed parts were designed using SolidWorks 2024 (Dassault Systems Deutschland GmbH, Germany) and printed using two different high resolution multi-jet 3D printers, the ProJet MJP 2500 Plus (3D Systems, USA) for printing of the polyacrylate VisiJet M2S-HT90 (3D Systems, USA) and the AGILISTA-3200 W (Keyence, Germany) for printing of the polyacrylate AR-M2 (Keyence, Germany) material, which was printed in two configurations, glossy and matte.

Post-processing of the VisiJet M2S-HT90 material included removal of the support material VisiJet® M2 Sup (3D Systems, USA) with the following steps: (1) detachment of the parts from the print platform after 10 min incubation at -20 °C, (2) incubation in a heat steam bath for 45 min, (3) incubation in a paraffin oil bath (15 min, 65 °C), (4) incubation in a ultrasonic paraffin oil bath (15 min, 65 °C), (5) 3x incubation in a ultrasonic bath with ddH_2O and detergent (15 min, 65 °C) and (6) incubation in an ultrasonic bath with ddH2O only (15 min, 65 °C).

Post-processing of the AR-M2 material was performed similarly without step (1-3). The two possible printing modes *glossy* or *matte* were selected by changing a single setting in the printer software Modelling Studio (Keyence, Germany).

The following methods of sterilization were selected for the experiment: autoclaving at 121°C for 30 min, and treatment with 96% ethanol (VWR, USA) in various combinations. Treatment with ethanol was carried out in an ultrasonic bath at 35°C for 1 h, followed by an evaporation phase. The printed parts were then placed in Phosphate Buffered Saline (PBS, Capricorn Scientific, Germany) at room temperature for 1 h, and dried.

4.2 Cell Culture Experiments

HUVEC were cultured in EGM-2 (Endothelial cell growth medium-2, PromoCell, C-22011) in a density of 6000 cells/cm^2 at 37 °C in a controlled environment of 5 % CO_2 and ≥ 95 % humidity. Culture medium was supplemented with 10% fetal calf serum

(FCS, Capricorn Scientific GmbH, Germany) and 0.5% Gentamycin (VWR, USA).

For biocompatibility testing, HUVEC were seeded in a density of $4.5 \cdot 10^5$ cells/cm^2 on a single slide of the 3D printing material inside a 48-well plate (Sarstedt, Germany) ($2.86 \cdot 10^5$/well) to reach an estimated cell confluence of about 80 % after 24 h cultivation. To fix the slides on the bottom of the plate a 3D-printed cylinder was plugged into the 48-well (s. Figure S1 in the Appendix). Each condition was performed in triplicate, with 48-well plates devoid of material slides serving as the control.

After cultivation, the cells were fixed for 30 min at RT with 4 % paraformaldehyde (PFA, VWR, USA) diluted in phosphate buffered saline (PBS) (Capricorn Scientific, Germany). Cell nuclei were stained with Hoechst 33342 dye (1:1000, Thermo Fisher Scientific, Germany). Actin filaments were stained with Phalloidin iFluor 555 Reagent-CytoPainter (abcam, GBR; diluted 1:1000 in PBS with 1% BSA (Sigma Aldrich Chemie GmbH, Germany)).

Cells were imaged using a Keyence BZ-X800 fluorescence microscope (Keyence, Germany) with a 20x objective. The observed imaging area was 0.004 cm². Cells were counted on three pictures of each triplicate.

4.3 Statistical Analysis

Each condition was performed in triplicates, with 48-well plates devoid of material slides serving as the control. Levels of significance were analyzed using one-way analysis of variance (ANOVA). Differences were considered as significant at $p < 0.05$. Significance levels were indicated with *p < 0.05, **$p < 0.01$ and ***$p < 0.001$.

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APPENDIX

Figure S1: CAD of the 3D-printed material slide (bottom) (9x1 mm) and cylinder (top) (9x15 mm) for the investigation of the endothelial cell adhesion.

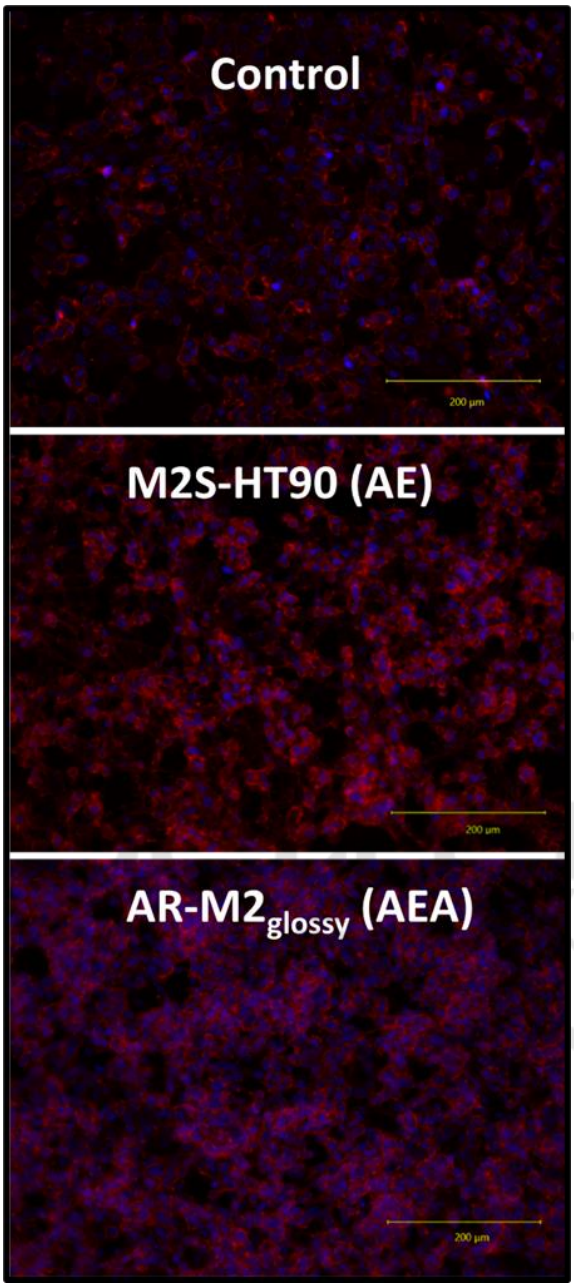

Figure S2: Representative images (20x objective) of phalloidin- and Hoechst 33342-stained HUVECs after 24 h cultivation on 48-well plates as control and M2S-HT90 and AR-$M2_{glossy}$ 3D-printed material slides at the best performing sterilization/disinfection procedures AE and AEA (A: autoclaving; E: ethanol disinfection).