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3D (bio)printing of lungs: past, present, and future

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Take Home Message Text: The field of 3D bioprinting is rapidly evolving, and the lung is not being left behind. Interdisciplinary advances in 3D printing technology, bioink formulations, and cell biology are moving closer towards faithfully recapitulating the native human lung.

Conflict of Interest Statement: All authors declare their submission of US Provisional Patent 63330075 – “Bioink for 3D Extrusion bioprinting, methods of making and uses thereof”

1 Introduction

To study processes important in lung health and disease, diverse pre-clinical models have been designed that vary in complexity and similarity to the *in situ* state[1]. Although two-dimensional (2D) systems are technically easy, they lack 3D microenvironment and dynamic forces, present in human lungs[1]. Animal models attempt to address these limitations but are themselves limited by divergence from human physiology[2].

Complex *in vitro* lung models have been developed to incorporate mechanical stretch and perfusion, demonstrating that these mechanical cues can alter cell proliferation, differentiation, migration, and immune responses[3]. Hydrogels, spheroids, and organoids are examples of static *in vitro* models that introduce complexity beyond traditional submerged 2D cultures. A hydrogel is a 3D cross-linked polymer that contains a significant amount of water and provides biomechanical/biochemical cues to cells. Tunability of the biomechanical/biochemical cues in 3D hydrogels are strengths of these models. Spheroids and organoids are most frequently static 3D tissue platforms, built from self-assembled cells alone (spheroids) or encapsulated in a hydrogel (organoid). Organoids are more complex and reproduce tissue characteristics such as cell-cell and cell-ECM interactions, cell organization, differentiation, and polarization. Lung-on-chip devices are particularly attractive because they can precisely engineer some cellular functions by leveraging microfabrication and microfluidic technologies. These micro-engineered cell culture systems can recapitulate some degree of complexity of human lung tissues by establishing air-liquid interface (ALI) and simulating breathing dynamics through the introduction of airflow and perfusion[5, 6] or

integrating a hydrogel layer for separating apical and basal compartments and promoting cell-ECM communication[7, 8].

3D bioprinting technology is emerging as a new standalone approach that also has the potential to merge and enhance existing model systems described above. 3D bioprinting technology aims to precisely control the spatial distribution of the 3D microenvironment for survival and function of multiple cell types. These model systems are presently being used for basic lung science while also being the foundation for future transplantation strategies[9]. This mini-review provides an overview of the 3D bioprinting field in the context of lung health and disease.

2 What is the status of 3D bioprinting technologies?

The creation of transplantable functional tissues and the development of *in vitro* tissue constructs for modeling diseases and drug screening has been the dominant focus in lung tissue engineering. Any engineered lung tissue should recapitulate the *in situ* functions and architecture with careful attention to the cellular and ECM composition. In general, 3D printing enables the creation of tangible objects from a computer-aided design file (Figure 1A.i) through a bottom-up manufacturing technique where materials are deposited on top of each other layer-by-layer. 3D bioprinting technologies facilitate this process by offering spatial control over where cells and biomaterials are deposited. Bioprinting requires that viable cell populations are suspended in "bioinks", a formulation able to keep cells healthy during automated fabrication that may or may not contain additional bioactive components and biomaterials. Diverse commercial and research lab developed bioinks are available, sharing core objectives of supporting cell viability and converting from a liquid ink into an immobile gel[4].

Inject, lithography, and extrusion bioprinting are the most frequently deployed technologies (Figure 1A.ii)[10]. Briefly, in inject bioprinting, bioink droplets are generated by thermal or piezoelectric processes that then deposit dropwise on a printing surface in a defined spatial location. Lithography-based technologies use light energy to cure bioinks at the desired locations. Extrusion bioprinting uses pressure through a syringe structure and needle-like nozzle for the delivery of a bioink. More information about these technologies can be found in recent reviews[9–11]. An adequate bioink should be non-cytotoxic and printable so that it does not impact cell viability before or after printing (Figure 1A.iii). A rapid change of the bioink from fluid to immobile gel can be facilitated by reactions between components in the bioink that cross-link upon exposure to chemical (e.g. free-radical initiators) or physical (e.g. temperature and pH) stimuli, in a process carefully timed to enable deposition on a printing surface (Figure 1B)[9].

Source of biomaterials for bioprinting can be natural biopolymers such as alginate, cellulose, collagen, gelatin, and decellularized ECM proteins, or synthetic polymers such as polycaprolactone and polyethylene glycol[4]. Although natural-based biopolymers provide biochemical cues for supporting cells, they have lower printability compared to synthetic polymers and may not transition from ink to gels efficiently[4]. To overcome this limitation, natural biopolymers have been chemically altered to add reactive functional groups (e.g. GELMA = gelatin + methacrylate groups) or composite hydrogels consisting of natural ECM and synthetic components have been developed[4]. Irrespective of the bioprinting method, there are broadly two approaches for introducing cells to the model: i) printing of a biomaterial ink without cells, in which cells are added post fabrication of a structure (Figure 1C.i) and ii) printing with cells, where cells are pre-mixed with a biomaterial in a bioink formulation (Figure 1C.ii)[9]. The first approach affords more flexibility over the printing

parameters (e.g. temperature, pressure, and cross-linking) that could impact cell viability. A limitation is that this approach focuses on cell seeding on the surface of the 3D printed model and may present challenges for long-term cell integration within the construct. The second approach that includes cells during the fabrication process enables cell deposition within a 3D printed microenvironment but is offset by printing parameters that could impact cell viability[9].

Current 3D bioprinting technologies may reach a resolution of ~ 100 microns[12], limiting the creation of alveolar-like constructs but enabling larger conducting airways constructs. The optimization of bioinks that enable viability of a myriad of lung cell types is ongoing, being performed in parallel with technology development that can improve the resolution of the 3D printing technology to scale down to the critical dimensions observed in the human lung. Despite 3D bioprinting's current resolution limitation, the technology does enable the incorporation of multi-cell types such as epithelial cells, endothelial cells, and fibroblast cells[13, 14].

2.1 3D bioprinting of artificial trachea

The initial use of 3D bioprinting for lung tissues focused on the trachea, in contrast to the more challenging distal airways and alveoli[15–17]. Trachea geometry can be achieved with the resolutions offered by current 3D printing technologies. The cartilage rings in the trachea have high mechanical strength, which is a desirable trait for 3D printing strategies as such structures can function as scaffolds for the deposition of softer materials and cells. The most recent technology has used an extrusion-based bioprinter with a dual-head printing strategy to simultaneously print stiff ring-shaped scaffolds and soft tissue-like constructs containing bioink and cells[17]. Artificial tracheas have been implanted in animals and showed epithelization and cartilage formation[17, 18].

2.2 3D bioprinting of cells at ALI

The respiratory mucosa and alveoli reside at an ALI that is required for epithelial cell differentiation[19]. 3D bioprinting is being applied to ALI culture models and has the potential to precisely control the composition and structure of the surface where cells grow to mimic the basement membrane for epithelium, which is not possible with conventional culture protocols. 3D bioprinted ALI lung tissue constructs have been developed that contain diverse lung cell types (alveolar type I and II cells, fibroblasts, and microvascular endothelial cells)[14, 20, 21]. 3D bioprinted ALI lung tissue constructs closely recapitulate the structure and function of native lung tissue and are therefore likely to find wide application in modeling lung health and disease[21].

2.3 Modeling lung infections and disease processes using 3D bioprinted constructs

In the context of lung infections, a Matrigel®-based bioink has been used to 3D bioprint a lung construct for studying influenza A infection[22]. The virus distribution, viral replication, infection pattern, and immune responses in these 3D bioprinted constructs were similar to those in native lungs, which could not be modeled in 2D cell culture. In the context of lung disease processes, a 3D bioprinting strategy has been leveraged to model lung cancer using a hydrogel bioink containing a suspension of A549 cancer cells in gelatin[23]. Unlike 2D cell culture models, these 3D bioprinted constructs enabled the study of both cell invasion and migration, which is crucial for studying cancer *in vitro*.

2.4 Alveoli-like 3D structures

Technical limitations in 3D bioprinting resolution and cell viability have hindered progress in creating alveoli-like 3D structures. Due to the dimensions of alveoli, no existing 3D bioprinting technologies can generate functional alveoli-like 3D structures with cells, although strategies are being pursued on scales larger than the human lung[24].

3 Challenges and future work

The lung is a complex and dynamic organ, making it challenging to model comprehensively *in vitro*. Any lung model should resemble the native structure, composition, dynamic environment, and mechanical properties. Among current approaches, 3D bioprinting is still in the early stages of development toward the long-term goal of building dynamic and functional *in vitro* lung models. Current 3D bioprinting technologies cannot offer the required resolution to print and model the small lung parenchyma tissues such as alveoli[25]. Furthermore, bioinks with cells are intrinsically heterogeneous and present challenges for controlled deposition of a particular cell in a specific location. However, the advancement in developing bioinks with tunable mechanical properties and bioactive compositions has proved that this technology offers many opportunities[26]. For instance, lung tissue implants can be constructed by optimizing the bioink composition in bioprinting so that the final product has the required mechanical strength, biocompatibility, and biodegradability. Similarly, when the focus is developing an *in vitro* lung construct to model a target disease or to study the impact of drug treatments, the bioink should be formulated based on the target's required mechanical and biochemical properties of lung parenchyma tissue. This means that the bioink should provide an environment that mimics the native lung microenvironment in health or disease. In this case, ECM-based hydrogels or composite hydrogels incorporating natural ECM proteins would be the ideal choice. Importantly, bioink compatibility with live cell fluorescence, immunohistochemistry, *in situ* hybridization, and histology stains must be addressed. Although live cell fluorescence imaging is possible, swelling of bioink-based constructs during histological processing methods may disrupt the original structure if fixation methods are not optimized using reagents designed to retain structure of unstable and viscous materials (e.g. HistoGel™).

Using sacrificial materials, which are used to provide structural support during printing and can be removed when printing of the desired structure is completed, can be a helpful strategy to create more complex 3D constructs while providing a cell-friendly microenvironment. For example, ECM-based hydrogels closely resemble the natural microenvironment of the cells, but they have constrained mechanical properties and are not always suitable for 3D bioprinting. The use of sacrificial biomaterials has improved the printability of these natural components. In one approach, the desired construct can be created in a pool of sacrificial material, which can be later removed without impacting the final product[27].

3D bioprinting with synthetic hydrogels offers advantages over native ECM but is limited by the absence of native cell-microenvironment interactions. Chemical modification of synthetic bioinks with the arginine-glycine-aspartate (RGD) sequence, an amino acid sequence within ECM components (e.g. fibronectin) that mediates cell attachment, can overcome such challenges by improving cell attachment, spreading, and proliferation[28]. However, these strategies have not yet been explored for creating *in vitro* lung models, and it is not fully understood how these modifications will impact the long-term culture of lung cells. Consistent with this approach, a tissue-specific hybrid bioink consisting of alginate and ECM derived from decellularized lung tissue has been introduced to improve mechanical properties and lung cell viability[29]. Therefore, there are opportunities to develop new bioinks and biomedical engineering tools to better model human lung tissues.

Future work needs to investigate how cells remodel and modify their original 3D bioprinted microenvironment and whether it evolves towards a more representative native microenvironment. The outcomes from these studies will define how the bioink composition should be altered to develop more physiologically relevant *in vitro* lung models.

4 Concluding remarks

The field of 3D bioprinting is rapidly evolving, and the lung is not being left behind. Interdisciplinary advances in 3D printing technology, bioink formulations, and basic lung cell biology will continue to move closer towards faithfully recapitulating the native human lung. Although the moonshot goal of a viable transplantable lung may still be far on the horizon, the lessons learned from the development and application of this technology are very likely to advance our understanding of lung health and disease.

Conflict of Interest:

Jeremy Hirota, Mohammedhossen Dabaghi, Mabel Barreiro Carpio and Jose Manuel Moran-Mirabal are listed as authors of provisional patent (US Provisional Patent 63330075 – “Bioink for 3D Extrusion bioprinting, methods of making and uses thereof”).

There are no further disclosures.

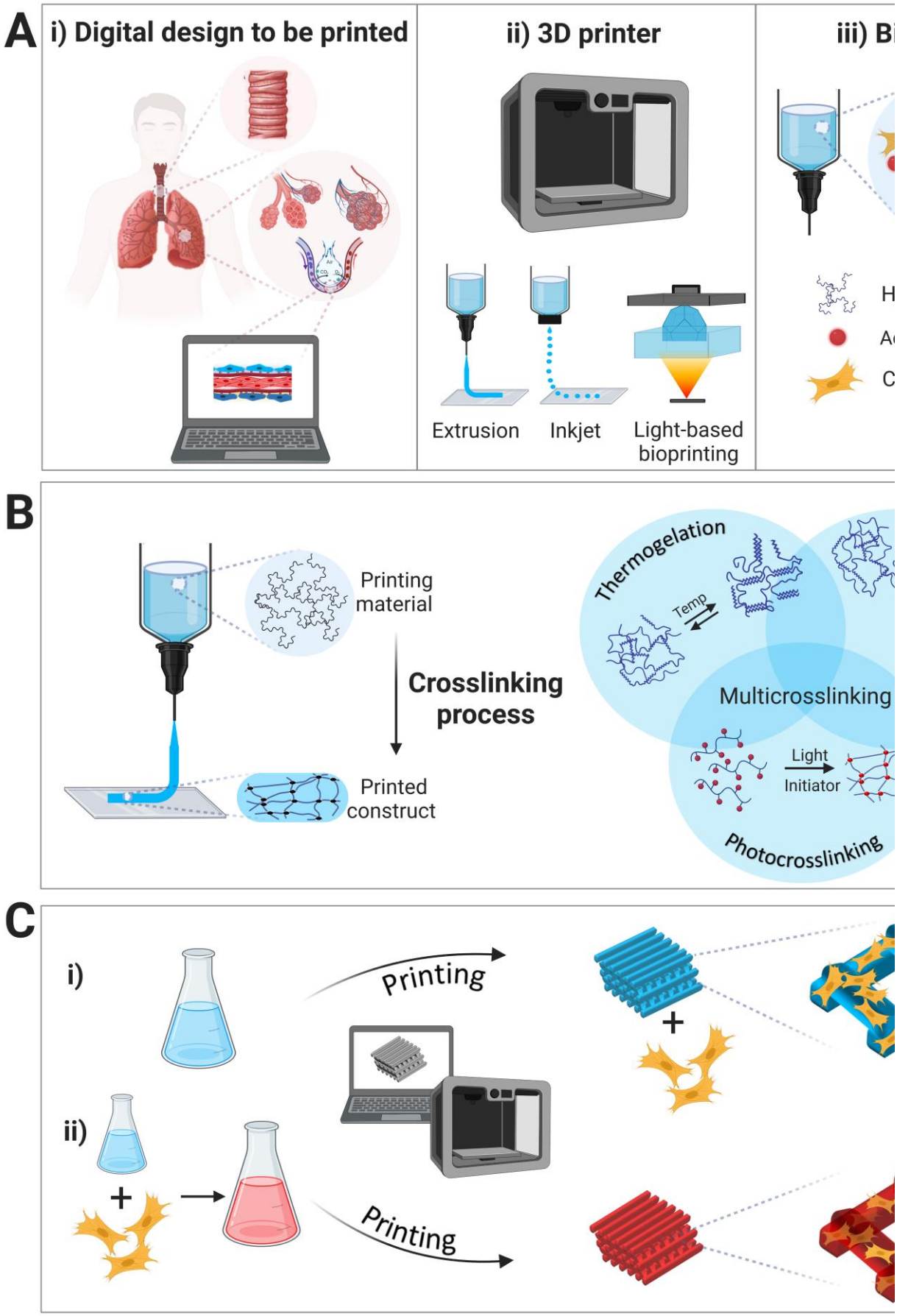

Figure 1: **3D Bioprinting for Modeling Lung Health and Disease. (A)** Resources and steps

required to 3D bioprint lung structures: **Left panel:** Digital designs of lung structures are inspired by the trachea, bronchi, and alveoli. **Middle panel:** Diverse 3D bioprinters are available that use different technologies to print, most frequently using extrusion, inkjet, or light assisted bioprinting technology. **Right panel:** The biomaterial or bioink, including cells, are the key components that will determine the properties and applications of the printed construct. **(B)** Various cross-linking strategies for bioink materials **(C)** Schematic representation of the bioprinting process: comparison of the two main approaches to bioprint, where the cells can be seeded post-fabrication in a biomaterial ink (**top panel**), or the cells are included during the fabrication process in a bioink (**bottom panel**). When cells are seeded after, they are not subjected to the stress associated to the printing process, but the cells will be only on the surface of the printed construct. On the other hand, when the cells are included in a bioink, they go through the printing process and will be dispersed in the hydrogel within the printed constructs. Figure created with Biorender.

5 References

1. Moreira A, Müller M, Costa PF, Kohl Y. Advanced In Vitro Lung Models for Drug and Toxicity Screening: The Promising Role of Induced Pluripotent Stem Cells. *Advanced Biology* [Internet] 2021 [cited 2022 Jan 17]; : 2101139Available from: https://onlinelibrary.wiley.com/doi/10.1002/adbi.202101139.

2. Ingber DE. Is it Time for Reviewer 3 to Request Human Organ Chip Experiments Instead of Animal Validation Studies? *Advanced Science* John Wiley and Sons Inc; 2020; 7.

3. Doryab A, Tas S, Taskin MB, Yang L, Hilgendorff A, Groll J, Wagner DE, Schmid O. Evolution of Bioengineered Lung Models: Recent Advances and Challenges in Tissue Mimicry for Studying the Role of Mechanical Forces in Cell Biology. Advanced Functional Materials Wiley-VCH Verlag; 2019.

4. Jorgensen AM, Yoo JJ, Atala A. Solid Organ Bioprinting: Strategies to Achieve Organ Function. Chemical Reviews American Chemical Society; 2020. p. 11093–11127.

5. Dongeun Huh, Benjamin D. Matthews, Akiko Mammoto, Martín Montoya-Zavala, Hong Yuan Hsin DEI. Reconstituting Organ-Level Lung. *Science (1979)* 2010; 328: 1662–1668.

6. Thompson CL, Fu S, Knight MM, Thorpe SD. Mechanical Stimulation: A Crucial Element of Organ-on-Chip Models. Frontiers in Bioengineering and Biotechnology Frontiers Media S.A.; 2020.

7. Humayun M, Chow CW, Young EWK. Microfluidic lung airway-on-a-chip with arrayable suspended gels for studying epithelial and smooth muscle cell interactions. *Lab on a Chip* Royal Society of Chemistry; 2018; 18: 1298–1309.

8. Xu C, Zhang M, Chen W, Jiang L, Chen C, Qin J. Assessment of Air Pollutant PM2.5 Pulmonary Exposure Using a 3D Lung-on-Chip Model. *ACS Biomaterials Science and Engineering* American Chemical Society; 2020; 6: 3081–3090.

9. Barreiro Carpio M, Dabaghi M, Ungureanu J, Kolb MR, Hirota JA, Moran-Mirabal JM. 3D Bioprinting Strategies, Challenges, and Opportunities to Model the Lung Tissue Microenvironment and Its Function. *Frontiers in Bioengineering and Biotechnology* Frontiers Media SA; 2021; 9.

10. Moroni L, Burdick JA, Highley C, Lee SJ, Morimoto Y, Takeuchi S, Yoo JJ. Biofabrication strategies for 3D in vitro models and regenerative medicine. Nature Reviews Materials Nature Publishing Group; 2018. p. 21–37.

11. Chen EP, Toksoy Z, Davis BA, Geibel JP. 3D Bioprinting of Vascularized Tissues for in vitro and in vivo Applications. Frontiers in Bioengineering and Biotechnology Frontiers Media S.A.; 2021.

12. Zheng Z, Eglin D, Alini M, Richards GR, Qin L, Lai Y. Visible Light-Induced 3D Bioprinting Technologies and Corresponding Bioink Materials for Tissue Engineering: A Review. Engineering Elsevier Ltd; 2021. p. 966–978.

13. Berg J, Weber Z, Fechler-Bitteti M, Hocke AC, Hippenstiel S, Elomaa L, Weinhart M, Kurreck J. Bioprinted multi-cell type lung model for the study of viral inhibitors. *Viruses* MDPI AG; 2021; 13.

14. Ng WL, Ayi TC, Liu Y-C, Sing SL, Yeong WY, Tan B-H. Fabrication and Characterization of 3D Bioprinted Triple-layered Human Alveolar Lung Models. *International Journal of Bioprinting* Whioce Publishing Pte Ltd; 2021; 7.

15. Park JH, Yoon JK, Lee JB, Shin YM, Lee KW, Bae SW, Lee JH, Yu JJ, Jung CR, Youn YN, Kim HY, Kim DH. Experimental Tracheal Replacement Using 3-dimensional Bioprinted Artificial Trachea with Autologous Epithelial Cells and Chondrocytes. *Scientific Reports* Nature Publishing Group; 2019; 9.

16. Taniguchi D, Matsumoto K, Tsuchiya T, MacHino R, Takeoka Y, Elgalad A, Gunge K, Takagi K, Taura Y, Hatachi G, Matsuo N, Yamasaki N, Nakayama K, Nagayasu T. Scaffold-free trachea regeneration by tissue engineering with bio-3D printing. *Interactive Cardiovascular and Thoracic Surgery* Oxford University Press; 2018; 26: 745–752.

17. Kaye R, Goldstein T, Grande DA, Zeltsman D, Smith LP. A 3-dimensional bioprinted tracheal segment implant pilot study: Rabbit tracheal resection with graft implantation. *International Journal of Pediatric Otorhinolaryngology* Elsevier Ireland Ltd; 2019; 117: 175–178.

18. Park JH, Ahn M, Park SH, Kim H, Bae M, Park W, Hollister SJ, Kim SW, Cho DW. 3D bioprinting of a trachea-mimetic cellular construct of a clinically relevant size. *Biomaterials* Elsevier Ltd; 2021; 279.

19. Michi AN, Proud D. A toolbox for studying respiratory viral infections using air-liquid interface cultures of human airway epithelial cells. *American Journal of Physiology - Lung Cellular and Molecular Physiology* American Physiological Society; 2021; 321: 263–279.

20. Horvath L, Umehara Y, Jud C, Blank F, Petri-Fink A, Rothen-Rutishauser B. Engineering an in vitro air-blood barrier by 3D bioprinting. *Scientific Reports* Nature Publishing Group; 2015; 5.

21. Kang D, Park JA, Kim W, Kim S, Lee HR, Kim WJ, Yoo JY, Jung S. All-Inkjet-Printed 3D Alveolar Barrier Model with Physiologically Relevant Microarchitecture. *Advanced Science* John Wiley and Sons Inc; 2021; 8.

22. Berg J, Hiller T, Kissner MS, Qazi TH, Duda GN, Hocke AC, Hippenstiel S, Elomaa L, Weinhart M, Fahrenson C, Kurreck J. Optimization of cell-laden bioinks for 3D bioprinting and efficient infection with influenza A virus. *Scientific Reports* Nature Publishing Group; 2018; 8.

23. Wang X, Zhang X, Dai X, Wang X, Li X, Diao J, Xu T. Tumor-like lung cancer model based on 3D bioprinting. *3 Biotech* Springer Verlag; 2018; 8.

24. Grigoryan B, Paulsen SJ, Corbett DC, Sazer DW, Fortin CL, Zaita AJ, Greenfield PT, Calafat NJ, Gounley JP, Ta AH, Johansson F, Randles A, Rosenkrantz JE, Louis-Rosenberg JD, Galie PA, Stevens KR, Miller JS. Multivascular networks and functional intravascular topologies within biocompatible hydrogels. *Science (1979)* [Internet] 2019; 364: 458–464Available from: http://science.sciencemag.org/.

25. Galliger Z, Vogt CD, Panoskaltsis-Mortari A. 3D bioprinting for lungs and hollow organs. Translational Research Mosby Inc.; 2019. p. 19–34.

26. Petrou CL, D'Ovidio TJ, Bölükbas DA, Tas S, Brown RD, Allawzi A, Lindstedt S, Nozik-Grayck E, Stenmark KR, Wagner DE, Magin CM. Clickable decellularized extracellular matrix as a new tool for building hybrid-hydrogels to model chronic fibrotic diseases: In vitro. *Journal of Materials Chemistry B* Royal Society of Chemistry; 2020; 8: 6814–6826.

27. Lee A, Hudson AR, Shiwarski DJ, Tashman JW, Hinton TJ, Yerneni S, Bliley JM, Campbell PG, Feinberg AW. 3D bioprinting of collagen to rebuild components of the human heart. *Science (1979)* [Internet] 2019; 365: 482–487Available from: https://www.science.org.

28. Hersel U, Dahmen C, Kessler H. RGD modified polymers: Biomaterials for stimulated cell adhesion and beyond. *Biomaterials* Elsevier BV; 2003; 24: 4385–4415.

29. de Santis MM, Alsafadi HN, Tas S, Bölükbas DA, Prithiviraj S, da Silva IAN, Mittendorfer M, Ota C, Stegmayr J, Daoud F, Königshoff M, Swärd K, Wood JA, Tassieri M, Bourgine PE, Lindstedt S, Mohlin S, Wagner DE. Extracellular-Matrix-Reinforced Bioinks for 3D Bioprinting Human Tissue. *Advanced Materials* Wiley-VCH Verlag; 2021; 33.