

Harnessing Tissue Engineering Approaches to Develop Advanced 3D Models for Respiratory Drug Development and Respiratory Regenerative Therapies

AUTHOR(S)

Luis Soriano

CITATION

Soriano, Luis (2022): Harnessing Tissue Engineering Approaches to Develop Advanced 3D Models for Respiratory Drug Development and Respiratory Regenerative Therapies. Royal College of Surgeons in Ireland. Thesis. <https://doi.org/10.25419/rcsi.19281566.v1>

DOI

[10.25419/rcsi.19281566.v1](https://doi.org/10.25419/rcsi.19281566.v1)

LICENCE

CC BY-NC-SA 4.0

This work is made available under the above open licence by RCSI and has been printed from <https://repository.rcsi.com>. For more information please contact repository@rcsi.com

URL

https://repository.rcsi.com/articles/thesis/Harnessing_Tissue_Engineering_Approaches_to_Develop_Advanced_3D_Models_for_Respiratory_Drug_Development_and_Respiratory_Regenerative_Therapies/19281566/1



RCSI

Harnessing Tissue Engineering Approaches to Develop Advanced 3D Models for Respiratory Drug Development and Respiratory Regenerative Therapies

Luis Soriano Gamba, MSc

School of Pharmacy and Biomolecular Sciences

Tissue Engineering Research Group, Department of Anatomy and Regenerative
Medicine

A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and
Health Sciences, RCSI University of Medicine and Health Sciences, in fulfilment of
the degree of Doctor of Philosophy

Supervisors

Prof. Sally-Ann Cryan, Prof. Fergal J. O'Brien and Dr. Cian O'Leary

2022

Thesis Declaration

I declare that this thesis, which I submit to RCSI University of Medicine and Health Sciences for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI University of Medicine and Health Sciences or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed:

A handwritten signature in black ink, appearing to read 'Luis', enclosed within a large, loopy oval stroke.

Scholar Number: 18130011

Date: 27th February 2022

Table of Contents

Thesis Declaration	2
Abstract	8
Acknowledgments	9
Publications and Presentations	12
List of Abbreviations	15
List of Figures	17
List of Tables.....	24
List of Equations.....	25
Chapter 1	26
1.1 Significance of Respiratory Diseases in Society	27
1.2 The Respiratory Tract and the Trachea.....	30
1.2.1 Biology of the Trachea	31
1.2.2 Biomechanics of the Trachea	34
1.3 Tissue Engineering and the Airways	37
1.3.1 Cell Sources for Respiratory and Trachea Tissue Engineering	37
1.3.2 Biomaterials for Respiratory and Trachea Tissue Engineering	41
1.3.3 Manufacturing Techniques	45
1.4 In Vitro Models of the Tracheobronchial Region for Drug Development.....	51
1.5 Tissue-Engineered Approaches for Tracheal Regeneration.....	56
1.5.1 Early Attempts at Tracheal Regeneration and Restoration	57
1.5.2 Pre-clinical Outcomes of Tracheal Tissue Engineering Approaches	58
1.5.3 Clinical Translation of Tissue-Engineered Tracheas.....	60
1.6 Thesis Objectives.....	63
Chapter 2	64
2.1 Introduction	65
2.1.1 Aims and Objectives.....	66
2.2 Materials and Methods.....	68
2.2.1 Materials	68
2.2.2 Scaffold Fabrication	68
2.2.3 Cell Culture.....	69
2.2.3.1 Cell Selection and Culture Media.....	69
2.2.3.2 Non-polarised Epithelial Culture	69
2.2.3.3 Polarised Epithelial Culture on Transwell® Inserts	70
2.2.3.4 Polarised Epithelial Culture on CHyA-B Scaffolds	70

2.2.4	Evaluation of Epithelial Barrier Integrity	71
2.2.5	<i>In vitro</i> Exposure	71
2.2.5.1	Reagent Selection and Cell Exposure Optimisation	71
2.2.5.2	Cell Toxicity Assays.....	73
2.2.5.3	Secretion of Pro-inflammatory Cytokines.....	74
2.2.6	Data Analysis	74
2.3	Results	75
2.3.1	Evaluation of Epithelial Barrier Integrity.....	75
2.3.2	Bacterial-mediated Toxicity and Inflammation	77
2.3.2.1	Optimisation of Bacterial Exposure Conditions	77
2.3.2.2	Cell Toxicity After LPS Exposure in 2D and 3D	79
2.3.2.3	Inflammatory Response to LPS Exposure in 2D and 3D.....	82
2.3.3	Drug-mediated Toxicity and Inflammation.....	84
2.3.3.1	Optimisation of Drug-mediated Exposure Conditions.....	84
2.3.3.2	Cell Toxicity after Bleomycin Exposure in 2D and 3D	86
2.3.3.3	Inflammatory Response to Bleomycin Exposure in 2D and 3D	89
2.4	Discussion.....	91
2.5	Conclusion.....	97
Chapter 3		98
3.1	Introduction	99
3.1.1	Aims and Objectives.....	102
3.2	Materials and Methods.....	103
3.2.1	Materials	103
3.2.2	Scaffold Fabrication	103
3.2.2.1	Collagen and Hyaluronic Acid (CHyA) Film Fabrication.....	103
3.2.2.2	Collagen and Hyaluronic Acid-bilayered (CHyA-B) Scaffold Fabrication.....	103
3.2.2.3	Chemical Cross-linking	103
3.2.3	Cell Culture.....	104
3.2.3.1	Cell Selection and Culture Media.....	104
3.2.3.2	Polarised Epithelial Monoculture on Transwell® Inserts.....	104
3.2.3.3	Polarised Epithelial Co-culture on CHyA-B Scaffolds	105
3.2.4	Evaluation of Epithelial Barrier Integrity.....	105
3.2.5	<i>In vitro</i> Exposure	106
3.2.5.1	Nanoparticle Selection and Preparation.....	106
3.2.5.2	Cell Toxicity	107
3.2.5.3	Secretion of Pro-inflammatory Cytokines.....	108

3.2.6	Data Analysis	109
3.3	Results	110
3.3.1	Evaluation of Epithelial Barrier Integrity	110
3.3.2	Short-term Toxicological and Inflammatory Assessment of Nanoparticles	111
3.3.2.1	Cell Toxicity after Nanoparticle Exposure in 2D and 3D	111
3.3.2.2	Inflammatory Response after Nanoparticle Exposure in 2D and 3D	114
3.3.3	Long-term Toxicological and Inflammatory Assessment of Nanoparticles	116
3.3.3.1	Cell Toxicity after Nanoparticle Exposure in 2D and 3D	116
3.3.3.2	Inflammatory Response after Nanoparticle Exposure in 2D and 3D	118
3.3.3.3	Barrier Integrity	120
3.4	Discussion	122
3.5	Conclusion	130
Chapter 4		131
4.1	Introduction	132
4.1.1	Aims and Objectives	134
4.2	Materials and Methods	136
4.2.1	Materials	136
4.2.2	Polyglobalide (PGL) Synthesis	136
4.2.3	3D Printing of Polyglobalide (PGL)	137
4.2.4	Post Printing Characterisation of Polyglobalide (PGL)	138
4.2.4.1	Construct Weight Evaluation	138
4.2.4.2	Mechanical Characterisation	138
4.2.4.3	Differential Scanning Calorimetry	139
4.2.4.4	Fibre Diameter and Quantification	139
4.2.5	NaOH and EDAC Treatment Effect on Polymer-CHyA Disc Scaffolds	139
4.2.6	Freeze-drying of Polymer-CHyA Disc Scaffolds	140
4.2.7	Pore Size Analysis of Polymer-CHyA Disc Scaffolds	140
4.2.8	Scaffold Ultrastructure Characterisation	141
4.2.9	Biocompatibility Assessment of PGL-D-CHyA Disc Scaffolds	141
4.2.9.1	Cell Selection and Culture Media	141
4.2.9.2	Cell Metabolic Activity	142
4.2.9.3	Lactate Dehydrogenase (LDH) Release	142
4.2.9.4	DNA quantification	142
4.2.9.5	Live/Dead Imaging	143
4.2.10	Statistical Analysis	143
4.3	Results	144

4.3.1	PGL Synthesis	144
4.3.2	3D Printing of Polyglobalide (PGL) polymers	146
4.3.3	Post-printing Characterisation of Polyglobalide (PGL) Lattice Constructs.....	148
4.3.4	Incorporation of 3D Printed PGL into CHyA Scaffolds	151
4.3.5	PGL-CHyA Scaffold Biocompatibility	155
4.4	Discussion.....	159
4.5	Conclusion.....	165
Chapter 5		166
5.1	Introduction	167
5.1.1	Aims and Objectives.....	170
5.2	Materials and Methods.....	171
5.2.1	Fabrication of PCL- and PGL-CHyA-B Scaffolds	171
5.2.1.1	Polyglobalide (PGL) Synthesis	171
5.2.1.2	Scaffold Design and 3D Printing	171
5.2.1.3	Preparation of Collagen and Hyaluronic Acid (CHyA) Slurry and Films	172
5.2.1.4	Freeze-drying of PCL and PGL-CHyA-B Scaffolds	172
5.2.1.5	Cross-linking and Sterilisation.....	173
5.2.2	Characterisation of PCL- and PGL-CHyA-B Scaffolds.....	173
5.2.2.1	Mechanical Characterisation	173
5.2.2.2	Scaffold Ultrastructure Characterisation	174
5.2.3	Epithelialisation on PCL- and PGL-CHyA-B Tubular Scaffolds	174
5.2.3.1	Cell Selection and Culture Medium	174
5.2.3.2	Seeding Density and Rotation Time Optimisation	174
5.2.3.3	Optimisation of Epithelialisation on PCL-CHyA-B Tubular Scaffolds.....	175
5.2.3.4	Epithelialisation on PCL- and PGL-CHyA-B Tubular Scaffolds	176
5.2.4	Statistical Analysis	176
5.3	Results	178
5.3.1	3D printing of PCL and PGL Tubular and C-shaped Backbones.....	178
5.3.2	Mechanical Characterisation of PCL- and PGL-CHyA-B Tubular and C-shape Scaffolds 180	
5.3.3	Scaffold Ultrastructure Characterisation	184
5.3.4	Seeding Conditions Optimisation on PCL-CHyA-B Tubular Scaffolds.....	186
5.3.5	Optimisation of Epithelialisation on PCL-CHyA-B Tubular Scaffolds.....	187
5.3.6	Epithelialisation on PCL- and PGL- CHyA-B Tubular Scaffolds	190
5.4	Discussion.....	192
5.5	Conclusion.....	198

Chapter 6	199
6.1 General Discussion	200
6.2 Future Work	210
6.3 Concluding Remarks.....	215
Bibliography	216

Abstract

Respiratory diseases and major airway trauma represent a global healthcare emergency with most treatment options providing symptomatic relief rather than curative treatment. In addition, they are associated with enormous socioeconomic burden. Respiratory tissue engineering commonly focuses on two broad areas: (i) the development of novel *in vitro* models for drug discovery and (ii) on artificial constructs for tissue repair and regeneration. The development of physiologically-representative tracheobronchial tissue analogues has the potential to improve the translation of novel inhaled therapies by accurately reflecting *in vivo* respiratory pharmacological and toxicological responses. On the other hand, effective restoration of extensive tracheal damage arising from cancer, stenosis, infection or congenital abnormalities remains an unmet clinical need in respiratory medicine. In this project, tissue-engineered (TE) collagen hyaluronic acid bilayered (CHyA-B) scaffolds were used to evaluate bacterial and drug induced toxicity and inflammation using Calu-3 bronchial epithelial cells and Wi38 lung fibroblast co-culture under air liquid interface (ALI) conditions as an *in vitro* model of the upper respiratory tract. After using lipopolysaccharide (LPS) and bleomycin to induce bacterial and drug responses *in vitro*; differential cell responses were seen between 2D and 3D airway models. Furthermore, the co-culture 3D model provided differential epithelial responses to a number of inhaled nanoparticles compared to 2D models. Based on our understanding of the cell-matrix interactions involved in supporting respiratory epithelial cell growth and differentiation, we sought to harness these CHyA-B scaffolds to develop tubular TE tracheal constructs. A 3D-printed polymer mesh of thermoplastic polymers with tailored architecture was fabricated and characterised to provide the tubular backbone of the construct. Polyglycolide (PGL), an advanced synthetic polymer that allows for post-processing surface modifications in comparison to commonly used polymers such as polycaprolactone (PCL), was employed as a 3D printable material. This was then covered with CHyA-B scaffolds using a freeze-drying process and Calu-3 epithelial cells were seeded onto its luminal film surface resulting in the formation of a partial epithelial layer. 3D printed PCL- and PGL-CHyA tubular scaffolds are promising biocompatible prototypes for tracheal regeneration with adequate mechanical properties supporting epithelial growth.

Acknowledgments

Firstly, I would like to acknowledge my supervisors during this incredible journey over the last few years. Specially, I would like to express my sincere gratitude to Sally Ann; thanks you so much for giving me the opportunity to pursue this PhD, in which I have learned so many things that will definitely help me both professionally and personally in the future ahead. Thank you for your constant patience, support, advice and encouragement throughout these last three years as well as for the reading, correcting and proof-reading of all abstracts, manuscripts and specially the Chapters of this thesis. Cian, thank you for your patience guiding me too through this PhD, for the proof-reading and for the technical advice you have been giving me (hopefully I will not be analysing TEER data in the future). Finally, thank you Fergal for all the input you have given me and insights on the project and for appreciating a respiratory project away from bone and cartilage regeneration.

During my time in RCSI, I had the opportunity to meet a whole bunch of nice people that undoubtedly made me had an incredible time in the labs. To my RCSI friends and office mates, thanks so much for the inspiration, for being always kind and supportive, for the enjoyable times and of course for late Friday pizza and drinks in the pub. Although the list is long and probably I will forget some of you (sorry in advance), I would like to thank Rita, Isabel, Chris, Domhnall, Arlyng, Lia, Tom, Nour, Ronaldo, Martin, Claudio, Adrian, Alan, Ian, Cian, Martyna, Donagh, Kulwinder, Pedro, Tara, Silvia, Joanna, Elizabeth, Katelyn, Irina, Hannah, Laura, Giorgia, Aida, Elena, and Virginia. Special thanks to everyone that has been part of the aliquots team for spending hours in those tissue culture hoods over nice chats and laughs.

A big thank to all the staff in RCSI for always helping when needed and thanks to the technical staff in the School of Pharmacy and Biomolecular Sciences and in TERG. I would specially like to thank Vinnie, Sarah and Patrick for keeping the labs going even when facing Brexit, Covid-19 or any kind of worldwide shortages that was happening that month. Thanks for always welcoming me into your office for a nice chat, hearing me complaining about almost everything with a smile always in your face. A special mention here to Brenton, thanks for all the support when imaging and being always there to lend a hand when needed.

To all the past and current members from the Cryan's Group, although I am far away from all your drug delivery and nanoparticle expertise, thanks very much for all the advice and support you guys have given me. Thanks to Joanne R, Sarinj, Katie, Ahmad, Derek, Elena, Smiljana and Alice. Special thanks to Joanne O., thanks for being an amazing desk buddy, for all your help and advice, and for always listening to my daily rants every morning when things were not working. Thanks so much for always having a positive attitude and finding a solution to any problem no matter how impossible to solve it looked to me, hopefully I have picked up some of that myself.

Without the use of novel polymers from the Heise Group, this PhD would not have been possible at all. Special thanks to Andreas for all the advice, support and insights on all chemistry and polymer matters. Thanks also to everyone teaching and assisting me in synthesizing PGL and for keeping a supply chain for me to use it: Viviane, Shona, Rob and Ruairi.

I would like to personally acknowledge Dr. Lemoine for being the best 3D printing godfather anyone can dream of having. Mark, thanks so much for all your continuous help from developing python codes, to listening to me while panicking when PGL was not behaving itself and for all the expertise and knowledge you have showed me with all the printers we have in the lab. But not just only that, thanks so much for teaching me everything I know about freeze-drying, mechanical testing, slurry making, reading my thesis, assistance during analysing the results and probably many more things I cannot remember right now. Best super user ever!

Special thanks to my tracheal partner in crime, Tehreem. Thanks for being such a supportive friend, for keeping an eye on me all time while I was running from experiment to experiment. I cannot thank you enough for all your help and for always knowing my protocols better than me. Thanks so much, it has been a pleasure to be on this team with you!

Could not forget to thank my dear Lauren, Rachael, Rachel and Cami. Thank you for all the laughs, drinks and dinners, and for being such supportive friends. Lauren, I will never forget the long days stuck in cell culture with you fighting Transwells, scaffolds and TEER measurements. Thanks so much for all your help during this PhD, will always be my TEER buddy. Thank you Rachel for all the nanoparticle expertise and of course for being an ELISA queen, I wish one day I can be as precise and excellent

as you are! Thanks to Rachael for taking me away to the mountains running away from the labs, although you know I will always prefer a hike with a clear path to follow!

We have come to the part where I must change to my mother tongue (sorry non-spanish speakers). Por último, pero no menos importante, gracias a mi fantástica familia por estar siempre apoyándome durante los últimos tres años. Me gustaría agradecer especialmente a mis padres por ser un ejemplo a seguir y por todo el apoyo recibido: Lola y Gerardo. Muchas gracias por siempre creer en mí y por brindarme la oportunidad de recibir la mejor educación posible. Esperemos que este sea el último título/grado/master/diploma que hago. Muchas gracias por siempre estar ahí, por llevarme de viaje y de vacaciones cuando más se necesitaba y por ser un pilar muy importante en mi vida. Mama, muchas gracias por ser el mejor ejemplo a seguir, por preocuparte siempre por mí y en definitiva por aguantarme durante estos locos años. Papa, muchas gracias por tu apoyo durante este doctorado, ¡al final los dos somos doctores! Muchas gracias también a mi hermano Marcos, con esta tesis ya no tienes excusa para no saber a qué me dedico. Muchas gracias también a mis tías Gloria, Maria y Concha por su continuo apoyo. Finalmente, muchísimas gracias a mis amigos, especialmente a Adriana O, Adriana A, Ester e Idoia por todas las cervezas, fiestas y cenas; y hacer que me siguiese sintiendo en casa cuando volvía a Pamplona. Adriana, muchísimas gracias por todos los cafés, los viajes y por ser un gran apoyo durante este doctorado.

I am also grateful to Science Foundation Ireland for funding this work under Grant 13/2073.

Publications and Presentations

Publications

O'Leary, C., **Soriano, L.**, Fagan-Murphy, A., Ivankovic, I., Cavanagh, B., O'Brien, F. J., & Cryan, S. A. (2020). The fabrication and in vitro evaluation of retinoic acid-loaded electrospun composite biomaterials for tracheal tissue regeneration. *Frontiers in bioengineering and biotechnology*, 8, 190.

Soriano, L., Khalid, T., O'Brien, F. J., O'Leary, C., & Cryan, S. A. (2021). A Tissue-Engineered Tracheobronchial In Vitro Co-Culture Model for Determining Epithelial Toxicological and Inflammatory Responses. *Biomedicines*, 9(6), 631.

Accepted for Publication in European Respiratory Review Journal: Development and Clinical Translation of Tubular Constructs for Tracheal Tissue Engineering: A Review. **Soriano, L.**, Khalid, T., Whelans, D., O'Hyallachain, N., O'Brien, F.J., O'Leary, C., Cryan, S.A.

Presentations

Poster Presentation: "An Electrospun Retinoic Acid-Loaded Scaffold as a Biomaterial for Tracheal Tissue Engineering". **L Soriano**, C O'Leary, A Fagan-Murphy, G Hendy, FJ O'Brien, and SA Cryan. 5th Annual Matrix Biology Ireland (MBI) Meeting, 21st to 22nd November 2018, Galway (Ireland).

Oral Presentation: "Retinoic-Acid Loaded Electrospun Scaffolds: a Novel Biomaterial for Tracheal Tissue Engineering". **Luis Soriano**, Cian O'Leary, Aidan Fagan-Murphy, Fergal O'Brien, Sally-Ann Cryan. 25th Annual Conference Bioengineering in Ireland (BinI), 18th to 19th January 2019, Limerick (Ireland).

Poster Presentation: "The Development of 3D Printed Scaffolds for Tracheal Tissue Engineering". **Luis Soriano**, Tehreem Khalid, Mark Lemoine, Fergal O'Brien, Andreas Heise, Cian O'Leary and Sally-Ann Cryan. Royal College of Surgeons in Ireland Research Day 2019, 7th March 2019, Dublin (Ireland).

Poster Presentation: "The Development of a 3D Tracheobronchial Co-culture Model Using Bilayered Scaffolds: a Platform for Respiratory Drug Development". Cian O'Leary, **Luis Soriano**, Barinedum Yorkuri, Brenton Cavanagh, Shirley O'Dea, Fergal

J. O'Brien and Sally-Ann Cryan. KEYSTONE Symposia Delivering Therapeutic Agents Across Biological Barriers (E1), 6th to 9th May 2019, Dublin (Ireland).

Oral Presentation: "A Novel Drug-Eluting Biomaterial for Tracheal Tissue Engineering". **Soriano, L.**, O'Leary, C., Fagan-Murphy, A., Hendy, G., O'Brien, FJ. and Cryan, SA. TERMIS European Chapter Meeting 2019, 27th to 31st May 2019, Rhodes (Greece).

Poster Presentation "The Development of a 3D Tracheobronchial Co-culture Model Using Bilayered Scaffolds: a Platform for Respiratory Drug Development". Cian O'Leary, **Luis Soriano**, Barinedum Yorkuri, Brenton Cavanagh, Shirley O'Dea, Fergal J. O'Brien and Sally-Ann Cryan. RCSI Lung Health Symposium 2019, 12th June 2019, Dublin (Ireland).

Poster Presentation "Assessing the Mechanical Properties of 3D Printed Tubular Scaffolds for Tracheal Regeneration". Tehreem Khalid, **Luis Soriano**, Mark Lemoine, Sally-Ann Cryan, Fergal J. O'Brien, Cian O'Leary. RCSI Lung Health Symposium 2019, 12th June 2019, Dublin (Ireland).

Oral Presentation: "The development of a tissue engineered in vitro model of the airway that provides physiologically-representative epithelial toxicological and inflammatory responses". **Luis Soriano**, Fergal J. O'Brien, Cian O'Leary, Sally-Ann Cryan. Controlled Release Society 2020 Annual Meeting and Exposition, June 27th to July 1st 2020, Las Vegas (USA).

E-Poster Presentation: "Development and characterization of an airway tissue engineered in vitro model to provide more physiologically-representative epithelial responses for the assessment of novel therapies". **Luis Soriano**, Fergal J. O'Brien, Cian O'Leary, Sally-Ann Cryan. American Thoracic Society (ATS) 2021 International Conference, May 14th to May 19th 2021, Online.

E-Poster Presentation: "A 3D Tissue-engineered Airway Model for the Assessment of Epithelial Responses to Nanomaterials". **Luis Soriano**, Rachel Gaul, Fergal J. O'Brien, Cian O'Leary, Sally-Ann Cryan. European Respiratory Society (ERS) 2021 International COngress International Conference, September 5th to September 8th 2021, Online.

Oral Presentation: “Characterisation and Epithelialisation of 3D Printed Scaffolds for Tracheal Tissue Engineering”. **Luis Soriano**, Ruairi P Brannigan⁴, Tehreem Khalid, Andreas Heise, Fergal J. O’Brien, Cian O’Leary, and Sally-Ann Cryan. Irish Thoracic Society (ITS) Scientific Meeting 2021, November 19th 2021. Online

Awards

RCSI CEO Innovation Award 2020 in recognition of identifying a potential commercial application for their research in 2020. (IDF: “Polyglobalide (PGL) as a versatile 3D printable biomaterial and its application in trachea tissue engineering”). RCSI University of Medicine and Health Sciences, Office of Research and Innovation.

Innovation Disclosure

“Polyglobalide (PGL) as a versatile 3D printable biomaterial and its application in trachea tissue engineering”. Sally-Ann Cryan, Cian O’Leary, Fergal J. O’Brien, Andreas Heise, **Luis Soriano**, Mark Lemoine, Tehreem Khalid, Ruairi Brannigan. (IDF Filed: RCSI, TTO: IDF2020-05).

List of Abbreviations

2D	Two Dimensional
3D	Three dimensional
AcOH	Acetic Acid
AFSC	Amniotic fluid Stem Cells
ALI	Air-liquid Interface
ANOVA	Analysis of Variance
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CF	Cystic Fibrosis
CHyA	Collagen and Hyaluronic Acid
CHyA-B	Collagen and Hyaluronic Acid Bilayered
CNTs	Carbon Nanotubes
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Coronavirus Disease
Đ	Dispersity
DC	Dendritic cells
DCM	Dichloromethane
DHT	Dehydrothermal
DPBS	Dulbecco's Phosphate Buffer Saline
DSC	Differential Scanning Calorimetry
dsDNA	Double Stranded DNA
ECM	Extracellular Matrix
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked Immunosorbent Assay
ES	Embryonic Stem Cells
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
GL	Globalide
GPC	Gel Permeation Chromatography
HBECs	Human Bronchoepithelial Cells
hMSCs	Human Mesenchymal Stem Cells
HMVEC-Ls	Human Lung Microvascular Endothelial Cells
HUVECs	Human Umbilical Vein Endothelial Cells
HyA	Hyaluronic Acid
IL-1β	Interleukin 1 β
IL-6	Interleukin 6
IL-8	Interleukin 8
iPSC	Induced Pluripotent Stem Cells
LDH	Lactate Dehydrogenase
LPS	Lypopolysaccharide
MMP	Matrix Metallopeptidases
M_n	Molecular number
MSCs	Mesenchymal Stem cells
M_w	Molecular weight
MWCNTs	Multi-Walled Carbon Nanotubes

NaOH	Sodium Hydroxide
NHBE	Normal Human Bronchoepithelial Cells
NHS	N-Hydroxysuccinimide
PAA	Poly(acrylic acid)
PBS	Phosphate Buffer Solution
PBT	Polybutylene Terephthalate
PCL	Polycaprolactone
PE	Polyethylene
PEEK	Polyether ether Ketone
PEG	Polyethylene Glycol
PEO	Poly(ethylene oxide)
PEOT	Polyethylene Oxide Terephthalate
PET	Polyethylene Terephthalate
PETE	Polyethylene terephthalate
PGA	Polyglycolic Acid
PGL	Polyglobalide
PLA	Polylactic Acid
PLGA	Poly(lactic-co-glycolic) acid
Pseudomonas Aeruginosa	P. Aeruginosa
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PVA	Poly(vinyl alcohol)
RGD	Arg-Gly-Asp
RT	Room Temperature
SARS-Cov-2	Severe Acute Respiratory Syndrome Coronavirus 2
SEM	Scanning Electron Microscopy
TBM	Tracheobronchomalacia
TE	Tissue-engineered
TEER	Transepithelial Electrical Resistance
TET	Tissue-Engineered Trachea
TNF-alpha	Tumour necrosis factor α
U.S.	United States

List of Figures

Figure 1. 1. The respiratory tract. (A) Schematic of the upper and lower portions of the respiratory tract. (B) Variety of tissues that form the trachea including the respiratory epithelium, cartilaginous rings, annular ligament, trachealis muscle and the tracheobronchial epithelium. Figures adapted from ^{34, 35} 31

Figure 1. 2. The tracheobronchial respiratory epithelium consists of three major cell type populations; goblet, ciliated and basal cells. Adapted from ⁵¹ 33

Figure 1. 3. Biomaterials for tissue engineering. (A) Characteristics and properties of synthetic and natural biomaterials for its use in tissue engineering applications. (B) Types of biomaterials and key factors used to classified biomaterials. Adapted from ^{125, 127, 128} 42

Figure 1. 4. Chemical structure of GL (A) and PGL (B). Adapted from ¹⁷¹ 45

Figure 1. 5. Techniques used for the manufacture of scaffolds for respiratory and tracheal tissue engineering applications. (A) 3D printing and bioprinting, (B) casting, (C) decellularisation and (D) electrospinning. Adapted from ^{176, 180-182} 46

Figure 1. 6. Models of the respiratory epithelium available. These in vitro models range from the cheap and high-throughput 2D and can increase in complexity and physiological relevance by addition of 3D and co-culture conditions to match physiological conditions similar to in vivo and ex vivo respiratory models. Adapted from ⁴ 54

Figure 1. 7. Examples of pre-clinical prototypes of tracheal grafts used in animal models for regeneration. (A) Collagen-coated nitinol frame implanted into a canine model showing cell engraftment and survival up to 18 months. (B) 3D bioprinted tracheal scaffold containing rabbit MSCs and rabbit epithelial cells using a 3D printed cylindrical shape made of PCL. Adapted from ^{220, 306} 60

Figure 1. 8. Examples of clinical translated strategies for tracheal repair in humans. (A) Marlex mesh tube reinforced with a propylene ring and coated with porcine dermal atelocollagen that after extensive in vitro and in vivo characterisation was used in a set of 4 patients for larynx and trachea repair. Follow up studies up to 34 months post-implantation demonstrated epithelialisation of the implanted graft and no stenosis observed³¹¹. (B) 3D printed airway splint using made of PCL for tracheal restoration. The 3D airway splint was 3D printed to develop a personalised medical device for airway collapse induced by TBM. After implantation and follow up assessment in 3 infants, no signs of TBM were detected showing a prolonged and successful airway growth²¹⁴. Images adapted from ^{214, 311} 62

Figure 2. 1. Evaluation of epithelial barrier integrity in air liquid interface (ALI) culture models used for exposure studies. (A) Calu-3 epithelial monocultures, Wi38 fibroblast monocultures, and Calu-3 Wi38 epithelial co-cultures on Transwell® inserts and CHyA-B scaffolds grown under ALI conditions for 14 days. (B) Average TEER values of Calu-3 epithelial cell barriers following plateau of electrical resistance (day 10, 12, and 14). (* TEER measurements for fibroblast monocultures on Transwell® inserts and CHyA-B scaffolds were performed as n = 2). Results displayed as mean ± SEM (n = 7). 76

Figure 2. 2. Barrier integrity from epithelial monocultures and co-cultures on Transwell® inserts and CHyA-B scaffolds. (A) TEER values measured at day 3, 5, 8, 10, 12, and 14 from Calu-3 epithelial monocultures (light green) and Calu-3 Wi38 epithelial co-cultures (dark green) on Transwell® inserts; and Calu-3 epithelial monocultures (light blue) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds under ALI conditions. (B) Average TEER values of epithelial cultures following plateau of electrical resistance measured at day 10, 12, and 14. Results displayed as mean $\pm$ SEM (n = 7; *p < 0.05, ** p < 0.01)..... 76

Figure 2. 3. Optimisation of LPS dose response in Calu-3 epithelial cells. (A) IL-8 secretion from non-polarised Calu-3 epithelial cells following LPS exposure with 10, 1, and 0.1 μ g/ml of *E. coli* and *P. aeruginosa* derived LPS. (B) IL-8 secretion from non-polarised Calu-3 epithelial cells following LPS exposure 10, 1, and 0.1 μ g/ml of *P. aeruginosa*-derived LPS in 1% (serum starvation) and 10% (no serum starvation) serum Calu-3 medium. (C) IL-8 secretion from polarised Calu-3 epithelial cells on Transwell® inserts following 10 μ g/ml of *P. aeruginosa* derived LPS for an exposure time of 24 and 48 h. Cytokine release was quantified in both apical and basolateral compartments after bacterial challenge. Results displayed as mean $\pm$ SEM (n = 1; * p < 0.05, ** p < 0.01, *** p < 0.001). 78

Figure 2. 4. Cell viability of epithelial monocultures and co-cultures grown on Transwell® inserts and CHyA-B scaffolds following bacterial challenge with 10 μ g/ml of *P. aeruginosa*-derived LPS for 24 h. Apical (A) and basolateral (B) cell metabolic activity of Calu-3 epithelial monocultures, Wi38 fibroblast monocultures and Calu-3 Wi38 epithelial co-cultures on Transwell® inserts and CHyA-B scaffolds. Apical and basolateral LDH release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 epithelial co-cultures (D) on Transwell® inserts and CHyA-B scaffolds following LPS exposure. Changes in metabolic activity and LDH release are relative to untreated samples for each culture condition. Results displayed as mean $\pm$ SEM (n $\geq$ 3). 80

Figure 2. 5. TEER measurements following LPS exposure. TEER recovery of Calu-3 epithelial monocultures (A) and Calu3 Wi38 epithelial co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following bacterial challenge with 10 μ g/ml of *P. aeruginosa*-derived LPS for 24 h. TEER recovery values normalised to the recovery of untreated cultures. Results displayed as mean $\pm$ SEM (n = 3)..... 81

Figure 2. 6. Pro-inflammatory cytokine release profile from epithelial cultures following LPS exposure. Apical and basolateral IL-8 release from Calu-3 monocultures (A) and Calu-3 Wi38 co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following LPS exposure with 10 μ g/ ml of *P. aeruginosa* derived LPS for 24 h. Apical and basolateral IL-6 release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 co-cultures (D) on Transwell® inserts and CHyA-B scaffolds following LPS exposure. Results displayed as mean $\pm$ SEM (n = 3; * p < 0.05, ** p < 0.01)..... 83

Figure 2. 7. Optimisation of drug-mediated challenge in polarised Calu-3 epithelial cells on Transwell® inserts with 1, 10, and 100 μ g/ml of bleomycin for a 24 h exposure time. (A,B) Apical and basolateral cell metabolic activity from polarised Calu-3 epithelial cells. (C) Basolateral IL-8 secretion from polarised Calu-3 epithelial cells. (D) TEER recovery from polarised Calu-3 epithelial cells on Transwell® inserts TEER recovery values normalised to the recovery of untreated samples. Results displayed as mean $\pm$ SEM (n = 1; * p < 0.05, ** p < 0.01, *** p < 0.001)..... 85

Figure 2. 8. Cell viability on Transwell® inserts and CHyA-B scaffolds following drug-mediated challenge. Apical (A) and basolateral (B) cell metabolic activity of Calu-3 epithelial monocultures, Wi38 fibroblast monocultures, and Calu-3 Wi38 epithelial co-cultures on Transwell® inserts and CHyA-B scaffolds following exposure to 100 µg/ml of bleomycin for 24 h and measured at day 23 in culture. Apical and basolateral LDH release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 epithelial co-cultures (D) on Transwell® Inserts and CHyA-B scaffolds following bleomycin exposure. Changes in metabolic activity and LDH release are relative to untreated samples for each culture condition. Results displayed as mean ± SEM (n ≥ 3; * p < 0.05). 87

Figure 2. 9. TEER measurements following drug-mediated challenge. TEER recovery of Calu-3 epithelial monocultures (A) and Calu3 Wi38 epithelial co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following challenge with 100 µg/ml of bleomycin for 24 h measured at day 15, 17, 19, 21, and 23 in culture. TEER recovery values normalised to the recovery of untreated samples for each culture condition. Results displayed as mean ± SEM (n = 3; * p < 0.05, ** p < 0.01). 88

Figure 2. 10. Pro-inflammatory cytokine release profile from epithelial cultures following drug-mediated challenge. Apical and basolateral IL-8 release from Calu-3 epithelial monocultures (A) and Calu-3 Wi38 co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following challenge with 100 µg/ml of bleomycin for 24 h measured at day 23 in culture. Apical and basolateral IL-6 release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 co-cultures (D) on Transwell® inserts and CHyA-B scaffolds following bleomycin exposure. Results displayed as mean ± SEM (n = 3; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001). 90

Figure 3. 1. Schematic of culture conditions used for Calu-3 epithelial monoculture on Transwell® inserts and Calu-3 Wi38 epithelial co-culture on CHyA-B scaffolds. 105

Figure 3. 2. Barrier integrity from epithelial monocultures grown on Transwell® inserts and epithelial co-cultures from CHyA-B scaffolds. (A) TEER values measured at day 3, 5, 8, 10, 12 and 14 from Calu-3 epithelial monocultures on Transwell® inserts (dark blue) and Calu-3 Wi-38 epithelial co-cultures on CHyA-B scaffolds under ALI conditions. (B) Average TEER values of epithelial cultures following plateau of electrical resistance measured at day 10, 12 and 14. Results displayed as mean ± SEM. (n=3)..... 110

Figure 3. 3. Apical (A) and basolateral (B) cell metabolic activity measured from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Results displayed as mean ± SEM (n=3, *p<0.05). 112

Figure 3. 4. Apical (A) and basolateral (B) LDH release from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Max LDH denotes epithelial cultures treated with Lysis Buffer acting as positive controls for LDH release. Results displayed as mean ± SEM (n=3, *p<0.05, ****p<0.0001). 113

Figure 3. 5. Apical (A) and basolateral (B) IL-8 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-8 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, **p<0.01). 115

Figure 3. 6. Apical (A) and basolateral (B) IL-6 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-6 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05). 115

Figure 3. 7. Apical (A) and basolateral (B) cell metabolic activity measured from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 23 of culture, 216 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Results displayed as mean $\pm$ SEM (n=3, *p<0.05). 117

Figure 3. 8. Apical (A) and basolateral (B) LDH release from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 23 of culture, 216 h after exposure. Max LDH refers to epithelial cultures treated with Lysis Buffer acting as positive controls for LDH release. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, ****p<0.0001). 117

Figure 3. 9. Apical (A) and basolateral (B) IL-8 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 23 of culture, 216 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-8 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). 119

Figure 3. 10. Apical (A) and basolateral (B) IL-6 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 23 of culture, 216 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-6 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, **p<0.01). 119

Figure 3. 11. TEER recovery of epithelial Transwell® monocultures (light) and epithelial CHyA-B co-cultures (dark) following exposure to LPS (A), bleomycin (B), PLGA nanoparticles (C), CNTs (D), G5 miR (E) and G5 NC (F) nanoparticles measured at day 15, 17, 19, 21 and 23 of culture following exposure. TEER recovery values were normalised to the recovery of untreated samples for each culture condition. Results displayed as mean $\pm$ SEM (n=3; *p<0.05, **p<0.01). 121

Figure 4. 1. Schematic designs used for 3D printing PGL constructs. (A) Lattice geometry used for optimizing PGL 3D printing parameters and post-printing characterisation. (B) Disc geometry used for reinforcing CHyA scaffolds to evaluate PGL biocompatibility.	138
Figure 4. 2. Gel permeation chromatography (GPC) from synthesised PGL: (A) PGL-A, (B) PGL-B, (C) PGL-C and (D) PGL-D. (E) M_n , M_w and dispersity of synthesised PGL-A, PGL-B, PGL-C and PGL-D.	145
Figure 4. 3. 3D printing optimisation of PGL. (A) Printing conditions used to 3D print PGL-A, PGL-B, PGL-C and PGL-D. (B) Macrographs of PCL and PGL printed constructs.	147
Figure 4. 4. Post printing characterisation of 3D printed PGL lattice constructs in comparison to PCL 37 kDa and 50 kDa. (A) Weight uniformity of the printed constructs (n=30). (B) Mechanical compression of 3D printed constructs using 20% Strain and 50N load cell. Results displayed as mean $\pm$ SEM (n = 6). (C) Differential scanning calorimetry. (*p<0.05, **p<0.01, ***p<0.0001).	149
Figure 4. 5. Post printing characterisation of 3D printed PGL lattice constructs in comparison to PCL 37 kDa and 50 kDa. (A) Mean fibre diameter of printed constructs. (B-E) Representative brightfield visualisation of printed constructs. Results displayed as mean $\pm$ SEM (n = 5, *p<0.05, **p<0.01, ***p<0.001).	150
Figure 4. 6. Effects of EDAC and NaOH treatment on the mechanical properties of PGL-D printed discs using 20% strain and 50N load cell. (A) Mechanical properties before and after EDAC treatment of the 3D printed disc scaffolds (n=5). (B) Mechanical properties of PGL 3D printed constructs treated with 3M NaOH for 4, 12, 24 and 48 h (n=3, **p<0.01).	152
Figure 4. 7. SEM imaging of (A) CHyA, (B) PCL-37-CHyA, (C) PCL-50-CHyA and (D) PGL-D-CHyA disc scaffolds using a 250X and 77X magnification.	153
Figure 4. 8. Pore size analysis of CHyA reinforced 3D printed discs: (A) CHyA, (B) PCL-37-CHyA, (C) PCL-50-CHyA (D) PGL-D-CHyA disc scaffolds. (E) Mean pore diameter of freeze-dried scaffold discs. Results displayed as mean $\pm$ SEM (n = 5).	154
Figure 4. 9. Cell metabolic activity measured from CHyA, PCL-37-CHyA, PCL-50-CHyA and PGL-D-CHyA disc scaffolds seeded with hMSCs. (A) Cell metabolic activity values measured at day 1, 7 and 14. (B) Normalised values to each the corresponding day 1 of each CHyA and polymer-reinforced CHyA scaffolds (n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). ...	156
Figure 4. 10. LDH release and DNA concentration of MSCs seeded onto CHyA, PCL-37-CHyA, PCL-50-CHyA and PGL-D-CHyA disc scaffolds. (A) LDH release measured in cell culture supernatant normalised to the secretion measured on CHyA scaffolds at day 1, 7 and 14 of culture. (B) DNA quantification measured at day 1, 7 and 14 of culture (n=3, ***p<0.001, ****p<0.0001).	157
Figure 4. 11. LiveDead stain using DAPI and ethidium homodimer-1 of hMSCs grown on CHyA, PCL-37-CHyA, PCL-50-CHyA and PGL-D-CHyA disc scaffolds seeded with human MSCs (n=2).	158

Figure 5. 1. Designs used to 3D print tubular constructs of PCL and PGL, consisting of 40 layers (0.25 mm each) of 4.8 and 6.6 mm inner and outer radius interconnected with 5 spokes in each layer. (A) Schematic view of tubular design and (B) c-shaped design comprising 288°. 172

Figure 5. 2. Custom stainless-steel mould use to freeze-dry tubular polymer-reinforced CHyA scaffolds (A) Upper plate, (B) lower plate and (C) mounted mould..... 173

Figure 5. 3. Representative images of PCL and PGL tubular and c-shape 3D printed backbone constructs comprising 40 layers of extruded polymer of 1 cm in height (PGL was printed using 55 PSI and 70 or 100°C and an extrusion speed of 350 mm/min while PCL used 90 PSI, 100°C and 260 mm/min). (A,B) Top and lateral view of PCL and PGL 3D printed constructs. (C) PGL tubular constructs 3D printed using 70 and 100°C. 179

Figure 5. 4. Representative images of PCL- and PGL-CHyA-B tubular scaffolds. 3D printed tubular PCL or PGL backbones (A) were combined with CHyA-B scaffolds (B) and freeze-dried to produce PCL- and PGL-CHyA-B tubular scaffolds..... 181

Figure 5. 5. Mechanical compression of CHyA and PCL- and PGL-CHyA-B tubular and c-shape scaffolds using a 50N load cell, 1 cycle and 15% strain. (A) Young Modulus in MPa after performing longitudinal compression and (B) peak load in N after performing radial compression. Results displayed as mean $\pm$ SEM (n=4, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). 182

Figure 5. 6. Average peak load in N of PCL and PGL-CHyA-B tubular and c-shape tubular and c-shape scaffolds mechanically assessed under cyclic loading using a 50N load cell and 15% strain. The average peak load was measured at cycle 1 and cycle 250. Results displayed as mean $\pm$ SEM (n=4, ***p<0.001). 183

Figure 5. 7. Pore size analysis of tubular tracheal scaffolds. Representative images of toluidine blue staining of (A) CHyA-B, (B) PCL-CHyA-B and (C) PGL-CHyA-B tubular scaffolds. (D) Mean pore diameter of CHyA-B and PCL/PGL-CHyA-B tubular scaffolds. Toluidine blue imaging shows collagen and hyaluronic acid (HyA) stained in dark blue. Results displayed as mean $\pm$ SEM (n=4, **p<0.01, ***p<0.001). 184

Figure 5. 8. SEM imaging of freeze-dried CHyA-B and PCL- and PGL-CHyA-B tubular scaffolds using a 250X and 77X magnification. Representative images of (A) CHyA-B, (B) PCL- and (C) PGL-CHyA-B tubular scaffolds. 185

Figure 5. 9. Cellular seeding optimisation on PCL-CHyA-B tubular scaffolds using Calu-3 cells. (A) Optimisation of dynamic cell seeding using three different cell densities, evaluated using AlamarBlue™ at day 2 and day 7. Density A = 5×10^5 cells/cm², Density B = 10×10^5 cells/cm² and Density C = 20×10^5 cells/cm² (n=2). (B) Optimisation of seeding time under rotation (0.5 rpm) using Density A. Results displayed as mean $\pm$ SEM (n=3, ***p<0.001). . 186

Figure 5. 10. Epithelial cellular growth of Calu-3 cells measured on of PCL-CHyA-B tubular scaffolds seeded with 5×10^5 cells/cm² for 4 h under 0.5 rpm. (A) Cell metabolic activity and (B) DNA content measured from the CHyA film of PCL-CHyA-B scaffolds at day 2, 7 and 10 of culture. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, ***p<0.001). 187

Figure 5. 11. Epithelialisation and cell coverage of the CHyA film of PCL-CHyA-B tubular scaffolds seeded with 5×10^5 cells/cm² Calu-3 cells for 4 h under rotation (0.5 rpm). (A) Representative images of epithelial coverage of the film at day 2, 7 and 10 of culture using DAPI (blue) and phalloidin (red) staining at 4X and 10X magnification. (B) Coverage of the film by epithelial cells (%) at day 2, 7 and 10 of culture. Results displayed as mean $\pm$ SEM (n=3, **p<0.01)..... 189

Figure 5. 12. Cellular growth on PCL and PGL-CHyA-B tubular scaffolds. Scaffolds were seeded with 5×10^5 cells/cm² Calu-3 cells for 4 h under rotation (0.5 rpm). (A) Cell metabolic activity from full tubular scaffolds (B) DNA content measured from the CHyA film of PCL and PGL-CHyA-B tubular scaffolds at day 10 of culture. Results displayed as mean $\pm$ SEM (n=3, *p<0.05). 190

Figure 5. 13. Epithelialisation and cell coverage of the film of PCL- and PGL-CHyA-B tubular scaffolds seeded with 5×10^5 cells/cm² Calu-3 cells for 4 h under rotation (0.5 rpm). Representative images of epithelial coverage of the film from PCL (A) and PGL (B) reinforced CHyA-B tubular scaffolds at day 10 of culture using DAPI (blue) and phalloidin (red) staining at 4X magnification. Representative images of epithelial coverage of the film from PCL- (C) and PGL- CHyA-B tubular scaffolds (D) at 4X magnification. (E) Coverage of the film by epithelial cells (%) at day 10 of culture. Results displayed as mean $\pm$ SEM (n=2, **p<0.01). 191

List of Tables

Table 1. 1. Summary of mechanical studies performed in different tracheal tissues and whole traches from different animal models. The mechanical test employed as well as the obtained modulus with the appropriate study reference are included.....	36
Table 1. 2. Airway epithelial cell populations available for respiratory and trachea tissue engineering.	40
Table 3. 1. Size, charge and working concentration used for each of the nanoparticles use to expose epithelial cultures.....	107
Table 4. 1. Manufacturing conditions used to synthesize PGL-A, PGL-B, PGL-C and PGL-D.	137
Table 5. 1. Epithelial seeding densities used for tubular polymer-reinforced CHyA-B scaffolds.....	175

List of Equations

Equation 2. 1. Calculation of TEER recovery values.	73
Equation 3. 1. Dispersity ($\mathfrak{D}$) calculation.	136
Equation 5. 1. Epithelial coverage calculation.....	176

Chapter 1

Introduction and Literature Review

Part of the work presented in this chapter has been published in the European Respiratory Review Journal as “*Development and Clinical Translation of Tubular Constructs for Tracheal Tissue Engineering: A Review*”.

1.1 Significance of Respiratory Diseases in Society

Airway diseases stand as one of the most common medical pathologies affecting society and have a considerable health and economic impact, with an overall estimated cost of €380 billion in the European Union alone. The burden of respiratory diseases on society not only results in disability and mortality, but also leads to large health costs in expenditure for prescribed drugs as well as hospital inpatient care¹. This includes the commonly known “big five”- the major lung diseases defined by the Forum of the International Respiratory Society, which comprises asthma, chronic obstructive pulmonary disease (COPD), pneumonia, lung cancer and tuberculosis². Worldwide, it is estimated that respiratory diseases lead to the death of approximately four million people annually. It is important to highlight the heterogeneity of the health burden and risk factors in the different regions of the world associated with respiratory diseases³. Respiratory diseases are most prevalent in high income countries. South Asia and sub-Saharan Africa have the lowest prevalence of these diseases, but the highest respiratory-associated mortality is found in south Asia⁴. While indoor air pollution is the leading cause for chronic respiratory diseases in low and middle income countries, tobacco stands as the key risk factor in high income regions⁵. Respiratory diseases are therefore in urgent need of improved treatments and prevention strategies in order to reduce the staggering burden they place on society. Initiatives focusing on decreasing ambient pollution and campaigns focused on smoking must be further strengthened while early diagnosis and better treatments are needed to reduce the spread and effects of respiratory diseases⁴. Moreover, it has been reported that funding for respiratory research is inadequate and inequitable, with research expenditure in this field lower in comparison to cancer research, or cardiovascular diseases³. For example, the United States (U.S.) National Institutes of Health allocated \$122 million for COPD research while spending \$220 million in cannabis-related research in 2019⁶.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has focused world attention on the field of respiratory medicine and in particular the impact of respiratory infectious diseases with the ongoing coronavirus disease (COVID-19) pandemic^{7, 8}. COVID-19 has resulted in a staggering death toll and reduction in overall life expectancy worldwide representing the most important public health crisis in the last century, but it has also had a seismic impact on the global economy^{9, 10}. The

substantial burden on healthcare systems across all countries affected, important domestic income reductions, rises in unemployment, reduced manufacturing activity and premature deaths are all attributable to COVID-19¹¹. In parallel, global research efforts have made possible the development of vaccines in record-time, which has highlighted the potential for science to address urgent, unmet clinical needs when appropriate funding and support from governments is available in combination with excellent, collaborative scientific research work¹².

The estimated market size for respiratory diseases has seen a recent growth in the last decades, as its annual growth rate is estimated to be between 4% and 6%¹³. For instance, a market size of \$40 billion is estimated alone for asthma and COPD treatments in 2017¹⁴. Corticosteroids, bronchodilators and inhalers predominately occupy this market, with little presence of novel drugs targeting respiratory pathologies. The probabilities of any drug targeting respiratory diseases reaching the clinic is believed to be as low as 3%, mainly arising from poor understanding of disease, inadequate modelling for safety and efficacy testing leading to poor *in vitro*-*in vivo* correlation as well as a lack of investment^{13, 15}. Moreover, current treatments focus on symptomatic relief and palliative effects rather than curing the underlying disease cause and/or replacing malfunctioning tissues¹⁶. Recently, there has been increased clinical and industrial interest in developing better treatments for airway diseases with the development of bioengineered therapeutics including protein-based, gene-based and tissue-engineered (TE) therapies¹⁷. The clinical translation of these novel bioengineered therapies is highly dependent on the availability of adequate platforms used for its development and testing¹⁸. For example, the development of engineered biomaterials has the potential to increase clinical application of these advanced treatments by providing platforms that closer mimic *in vivo* lung physiology, allowing researchers to better engineer therapeutic delivery and assessing the effect they exert in the tissues of interest¹⁹. Although well-characterised platforms are used in drug delivery applications, they commonly suffer from poor correlation between *in vitro* and *in vivo* responses and fail to recapitulate human physiology when animal testing is included during pre-clinical assessment²⁰. Interestingly, this can be solved by implementing advanced multicellular platforms that recapitulate the three dimensional (3D) environment of the airways. Research focusing on the development of novel biomaterials and biomedical engineering offers a platform for the development

of these advanced bioengineered models by combining material science, drug delivery and pharmacology²¹. On the other hand, there is great interest in further deepening the knowledge on the regenerative capacities of the airways for repair using previous engineered biomaterials and cell-based strategies. Building on the work in developing advanced 3D models of the airways, this TE technology can be further harnessed for the development of treatment strategies aimed at airway repair. Nowadays, the treatment of long defects of portions of the airway is very restricted due to the lack of bioengineered grafts that allow for the restoration and regeneration of tracheal tissues. The combination of biomaterials, stem cells and growth factors has the potential to bridge the gap in airway regeneration and thereby restoring the functionality of damaged tissues arising from airway defects²²⁻²⁴.

Overall, the need for developing advanced strategies for better treatments and models of respiratory diseases is the main aim of the work presented throughout this thesis. The development of advanced *in vitro* models of the airway that provide relevant physiological responses to act as strong tools to boost the clinical translation in respiratory medicine is here investigated using bioengineered materials to support the formation of airway tissues¹⁹. At the core of a number of these advancements is the evolution of TE based technologies both as tools for translation and as medical devices. The combination of TE strategies with next generation material technologies such as 3D printing and electrospinning is starting to emerge as a promising field for the generation of airway substitutes repairing damaged tissue with the potential to significantly improve treatment of airway defects²⁵.

1.2 The Respiratory Tract and the Trachea

The respiratory system is divided into two functionally distinct zones; the conducting and the respiratory zones. The conducting region of the respiratory tract is responsible for the conditioning of the inhaled air as well as its transport to the respiratory region and includes the nose, nasopharynx, trachea and bronchioles. The respiratory region is responsible for gas exchange, begins from the bronchioles and comprises the alveolar ducts, alveolar sacs and the alveoli^{26, 27}. The respiratory system is also divided into two anatomically different sections; the upper respiratory tract from the nose to the larynx and the lower respiratory tract from the trachea to the alveoli (Figure 1.1A)²⁸. The respiratory system, which develops through prenatal and postnatal life, is highly complex with extensive cellular diversity. The respiratory epithelium in the lining of the conducting portion of the airways comprises several cell types and is surrounded by vascular and lymphatic conduits. It allows for the gas exchange process in the alveolar region, which contains an heterogeneous population of alveolar cells²⁹.

The tracheobronchial region of the respiratory tract is of crucial importance, as its inner lumen is formed by the respiratory epithelium with important physiological functions. The respiratory epithelium is responsible for the formation of the mucociliary escalator, which moves the respiratory mucus out of the respiratory tract expelling foreign particles and pathogens before they reach the lungs³⁰. This region of the respiratory tract is key during the process of drug discovery and development, as it is estimated that around 25% of inhaled particles deposit in the tracheobronchial epithelium³¹. Moreover, the tracheobronchial region is often subjected to severe trauma, which has prompted the development of multiple techniques and approaches aimed at tracheal tissue replacement and regeneration^{32, 33}.

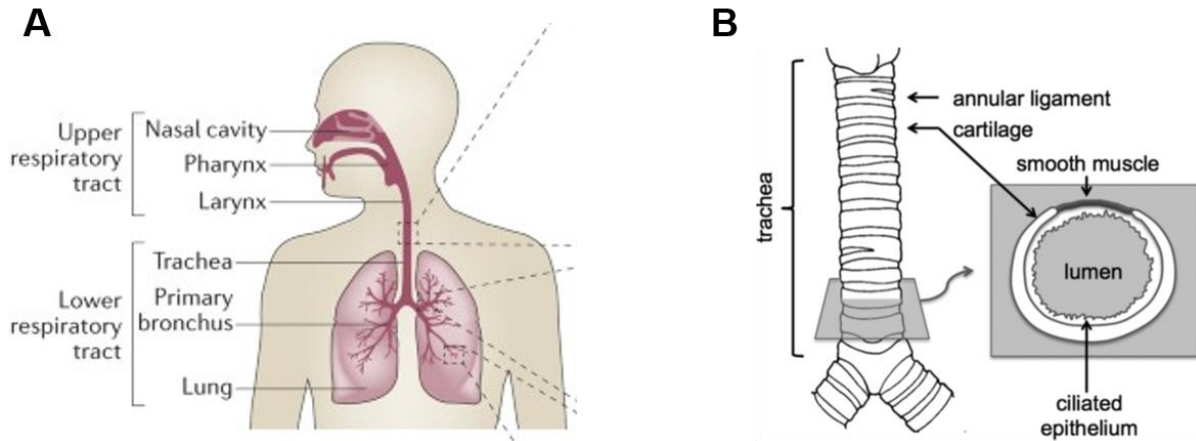


Figure 1.1. The respiratory tract. (A) Schematic of the upper and lower portions of the respiratory tract. (B) Variety of tissues that form the trachea including the respiratory epithelium, cartilaginous rings, annular ligament, trachealis muscle and the tracheobronchial epithelium. Figures adapted from ^{34, 35}.

1.2.1 Biology of the Trachea

The trachea is a hollow organ connecting the larynx and the main bronchi that serves as a conduit allowing the flow of oxygen to the lungs and the expiration of carbon dioxide during respiration³⁶. It is a flexible tube-like structure that bifurcates into the two main bronchi, which further subdivides into multiple smaller bronchi and 23 bronchioles generations, and it is also responsible for the humidification of inspired air and mucociliary clearance³⁷. The trachea is formed by 20 c-shape hyaline cartilaginous rings that comprise around 80% of the tracheal circumference and are connected in the longitudinal direction by the annular ligament (Figure 1.1B)³⁵. Both ends of each c-shape ring are connected together by the trachealis muscle, a smooth muscle tissue responsible for maintaining patency by increasing the diameter of the trachea during respiration, coughing and asthma^{35, 38}. The tracheal cartilage rings are not confluent to each other, being separated by the annular ligaments of the trachea³⁹. The inner lumen of the trachea is lined with a ciliated pseudostratified epithelium consisting mostly of goblet and ciliated cells⁴⁰. Anatomically, the trachea is divided into four main layers. The outermost layer, the adventitia, represents a collection of connective tissues connecting the trachea to the surrounding tissues. The cartilage rings, interconnected by the annular ligaments and the trachealis muscles, form the musclocartilaginous layer. Beneath this, the submucosa layer harbours blood vessels and nervous tissue while providing the trachea with support and elasticity due to the content of collagen and elastin fibres. Finally, the inner layer of the trachea

consists of the respiratory epithelium resting on a basement membrane and contains ducts connecting to the submucosal glands in the submucosa layer^{36, 41, 42}.

Blood supply to the trachea follows a longitudinally segmental pattern, in which arteries branch superiorly and inferiorly forming anastomosis with other contiguous arteries³⁶. This branching out into further small vessels allows blood supply to reach the epithelium passing between the cartilage rings⁴³. The inferior thyroid arteries arising from the subclavian arteries are responsible for the blood irrigation to the upper section of the trachea, while the lower section gets irrigation from the bronchial arteries derived from the aorta³⁶. Innervation of the trachea is achieved via the vagus and laryngeal nerves⁴⁴.

1.2.1.1 The Tracheobronchial Epithelium

The inner section of the tracheal tube is lined with respiratory epithelium. This ciliated pseudostratified columnar epithelium is responsible for mucociliary clearance and the moistening of the airways. It plays a crucial protective role, as it acts as a physical barrier against inhaled pathogens and irritant substances while being involved in the attraction of inflammatory cells during airway injury⁴⁵. The respiratory epithelium also plays an important role in lung immunity. It is responsible for the secretion of reactive oxygen and nitrogen species to eliminate inhaled pathogens and regulates the production of numerous cytokines for the recruitment of inflammatory cells⁴⁶.

The tracheal respiratory epithelium contains three major cell populations: ciliated columnar epithelial cells, goblet cells and basal cells (Figure 1.2). Ciliated cells are derived from basal or secretory cells and represent half of the total cell population of the respiratory epithelium, projecting 300 extensions per cell to the apical side, called cilia⁴⁵. Motile cilia on the apical surface of the respiratory epithelium measure between 5 and 10 μm and are responsible for the transport of liquid from the lungs towards the upper section of the respiratory system²⁷. Goblet cells, a specialised type of secretory cells, contain secretory vesicles that release high molecular weight mucins, mainly mucin 5AC and mucin 5B⁴⁷. The production of these surfactants in the lumen of the tracheal walls forms a layer of airway surface liquid called mucus. Goblet cells are polar cells with a nucleus located near the basement membrane containing the mucin rich granules close to the apical surface⁴⁸. Basal cells, located on the basal surface

attached to the thick respiratory epithelium basal membrane, are considered the stem cell population that act as progenitors for both ciliated and goblet cells in the tracheobronchial epithelium. This stem cell population is characterised by the expression of the transcription factor TP63 and cytokeratin 5; and plays a crucial role in tissue homeostasis and epithelium regeneration after injury⁴⁹. Basal cells also give rise to club or Clara cells, localised in the bronchioles, which can also act as progenitor cells crucial for epithelial repair⁵⁰.

The simplistic view of the airway epithelium as a set of three clearly defined and distinct cell populations has been recently challenged with the inclusion of advanced sequencing techniques⁵¹. New cell populations have been described, including tuft or brush cells; chemosensory cells believed to be involved in mediating the communication between neuronal and immune pathways⁵². Pulmonary ionocytes, recently identified and believed to arise from basal and tuft cells, are now regarded to play an important role in cystic fibrosis (CF), an inherited disease causing severe lung damage⁵³. In the surface of the tracheobronchial epithelium, pulmonary neuroendocrine cells are hypothesised to serve as chemosensors, eliciting immunological and physiological responses⁵¹. Further cell populations including hillock and microfold cells have been detected in the airway epithelium, albeit their role has yet to be fully understood. Finally, a wide variety of immune cells can be found underneath the respiratory epithelium, with an immune role against viral and bacterial infection via recruitment of T cells, B cells as well as natural killer cells⁵².

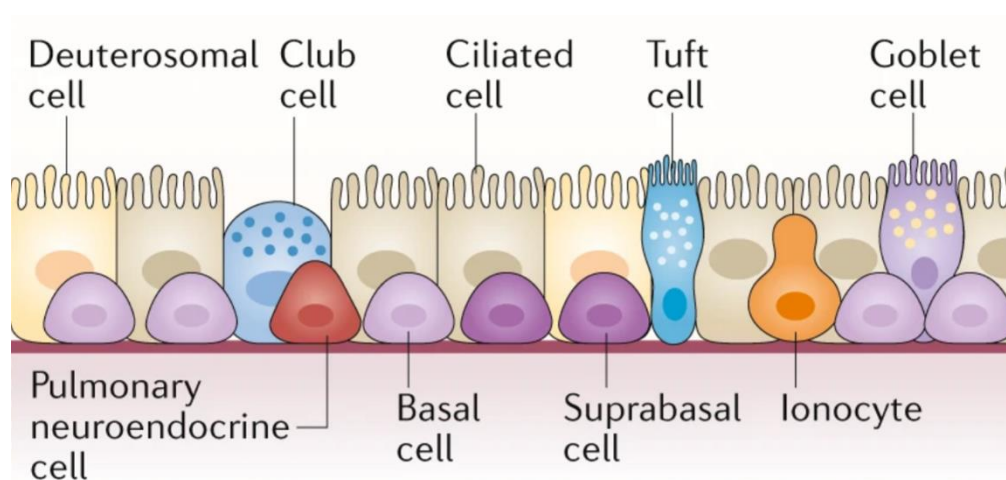


Figure 1. 2. The tracheobronchial respiratory epithelium consists of three major cell type populations; goblet, ciliated and basal cells. Adapted from ⁵¹.

1.2.1.2 Hyaline cartilage

Tracheal hyaline cartilage is mainly composed of chondrocytes and extracellular matrix (ECM). Cartilage ECM is characterised by the expression of collagen type II, III, V, VI and VI; and by a high content of proteoglycans including the predominant aggrecan⁵⁴. Type II collagen accounts for more than 90% of the total amount of collagen present in comparison to fibrocartilage, where type I is the most common⁵⁵. The content of the ECM dictates the properties of hyaline cartilage, with water and proteoglycans providing strength against compression forces while the network of collagen fibres provides tensile strength⁵⁶. Chondrocytes, embedded in lacunae structures in the hyaline ECM, have a crucial role in the organisation of the cartilage ECM. However, it is important to highlight the low capacity of hyaline cartilage for regeneration, which can be explained due to the lower blood supply in comparison to bone^{57, 58}. The main function of the consecutive hyaline rings forming the tracheal wall is maintaining the airway open to allow airflow and withstand forces exerted during respiration⁵⁹.

1.2.1.3 Trachealis Muscle and Annular Ligaments

The tracheal cartilage rings are interconnected by the annular ligaments, sections of fibroelastic tissue⁴¹. They present high anisotropic behaviour and, together with tracheal cartilage, are responsible for the trachea extensibility across the longitudinal axis while maintaining lateral rigidity³⁵. On the other hand, the trachealis muscle joins tracheal cartilage posteriorly and is composed of smooth muscle cell fibres⁶⁰. The main function of the trachealis muscle is to alter the tracheal diameter in response to coughing. Moreover, its contraction prevents the compression of the trachea allowing airflow during forced breathing situations^{35, 61}.

1.2.2 Biomechanics of the Trachea

The mechanical forces acting on tracheal tissues are numerous and complex to determine, as abnormal situations including forced respiration, coughing and sneezing exert changes in its physical properties such as the reduction of the tracheal cross section, increased radial deformation and stretching of the trachealis muscle³⁵. Moreover, the different components of the trachea possess distinct mechanical properties and therefore are evaluated as different materials. For instance, tracheal

cartilage is commonly studied as a biphasic material corresponding to the collagen rich solid matrix and the interstitial fluid⁶², but has also been assumed to have both linear and non-linear elastic behaviour⁶³. On the other hand, the trachealis muscle has been modelled as a hyperelastic material due to its non-linear mechanical properties and is stiffer longitudinally than circumferentially^{35, 63, 64}.

Table 1.1 outlines a summary of testing methods and specimens used for assessing the mechanics of this organ, as well as the mechanical strength observed in each study. Using compressive mechanical testing, human airway walls were reported to have a Young's Modulus in the range of 0.17-1.65 MPa and 0.25-2.2 MPa for tracheal cartilage⁶⁵. Animal models have commonly been used to study the mechanical properties of tracheal tissues. Compressive testing in pig tracheas reported a Young's Modulus of 1.36 ± 0.49 MPa and a Young's Modulus of 17.42 ± 12.24 MPa was reported for lamb tracheas^{66, 67}. Tensile testing on adult human tracheas reported an equilibrium modulus in the range of 5.05-39.63 MPa, 1-15 MPa, 4.6 ± 1.7 MPa and a Young's modulus of 3.33 MPa using an uniaxial tensile testing set up^{63, 68-70}. Ring bending mechanical studies have been also carried out to evaluate native mechanical properties reporting a Young's modulus of 2.5-7.7 MPa⁷¹. Due to the high variability in the animal model experimental set-up and the different mechanical properties that were under study, definition of a specific value for the mechanical properties of the trachea remains challenging. The use of different testing methods and the variability in the type of specimen employed hampers the development of engineered constructs with similar biomechanical properties to native tracheal tissues and still represents a challenge in the clinical translation of tracheal grafts⁷².

Table 1. 1. Summary of mechanical studies performed in different tracheal tissues and whole tracheas from different animal models. The mechanical test employed as well as the obtained modulus with the appropriate study reference are included.

	Specimen Type	Mechanical Test	Modulus
Cartilage	Human	Tensile	16.92 MPa ⁶³
	Human	Tensile	1-15 MPa ⁶⁸
	Porcine	Tensile Compression	5.62MPa 1.36 MPa ⁶⁶
	Ovine	Tensile	10.6 MPa ⁷³
	Ovine	Tensile Compression	33.50 MPa 17.42MPa ⁶⁷
Trachealis Muscle	Human	Tensile	15-1000 KPa ³⁸
	Human	Tensile	0.84-34.15 KPa ⁷⁰
Whole Trachea	Human	Tensile	12-20 MPa ⁶³
	Human	Compression	0.16-2 MPa ⁶⁵
	Porcine	Compression	1.10 MPa ⁶⁶
	Ovine	Tensile	1.5 MPa ⁷⁴
	Rabbit	Compression	1.72 MPa ⁷⁵
	Rabbit	Three-point bending	0.214 MPa ⁷⁶

1.3 Tissue Engineering and the Airways

The field of tissue engineering is experiencing an exponential growth over the last decades, focusing on regenerative therapies through the combination of different disciplines that include stem cell biology, biomaterials and nanobiotechnology among others⁷⁷. However, over the recent years, tissue engineering is not solely focused on regenerative medicine applications, but has broadened to include the development of *in vitro* platforms to better recapitulate tissue physiology and dynamics and thereby improve *in vitro* disease modelling and the *in vitro* tools available for drug development⁷⁸. The multidisciplinary field of tissue engineering is based in three main pillars; cells to form a functional matrix, scaffolds to support the growth of the cells mimicking the ECM and bioactive molecules in order to achieve tissue-like structures^{79, 80}. Respiratory tissue engineering, although not yet as developed as the fields of bone, cartilage or skin tissue engineering, has seen key advances over the last decade by focusing on two main areas. Firstly, the development of artificial platforms as *in vitro* models to better understand airway pathophysiology and serve as advanced platforms for the discovery, testing and validation of novel therapies for airway diseases¹⁶. These *in vitro* models of the airway have now been employed to establish 3D disease models to increase knowledge of the specific disease represented as well as to evaluate potential treatments⁸¹. Secondly, the use of TE grafts can be employed for the replacement and/or regeneration of airway tissues with lung and trachea tissue engineering being the most explored applications up-to-date^{73, 82}.

1.3.1 Cell Sources for Respiratory and Trachea Tissue Engineering

The cell population used in respiratory tissue engineering depends on the final application being considered and the tissue being regenerated or recapitulated, but airway epithelial cells are the most common cell type used (Table 1.2). Airway cell lines are mostly used when developing TE *in vitro* models, which are of immortalised nature and easily available⁸³. On the other hand, airway primary cells, with reduced proliferation and availability versus cell lines, are preferred for regenerative applications^{84, 85}.

Respiratory cell lines can be grouped in tracheobronchial and alveolar epithelial cells. Tracheobronchial cells are commonly used for applications focused on the conductive

part of the lower respiratory system, which includes the trachea and the bronchi. Calu-3 cells are the gold standard cell population for investigating the tracheobronchial respiratory epithelium⁸⁶. Calu-3 cells originate from human lung adenocarcinoma and form confluent and polarised epithelial monolayers with the formation of tight junctions, mucus secretion and high values of transepithelial electrical resistance (TEER)⁸⁷. Moreover, Calu-3 can be grown on permeable inserts under air-liquid interface (ALI) conditions which results in its widespread use in drug transport studies, drug targeting, aerosol studies and drug-drug interactions⁸⁸. 16HBE14o–, generated from an infant heart-lung cancer patient, are immortalised bronchial cells showing specific epithelial functions such as tight junction formation and display vectoral ion transport⁸⁹. 16HBE14o– cells also report adequate TEER values and the formation of tight junctions, being routinely used to model respiratory diseases (e.g. CF) and study barrier function, but do not form epithelial monolayers and have limited mucociliary properties in comparison to Calu-3 cells^{16, 90}. Another bronchoepithelial cell line available is BEAS-2B, a non-tumourgenic cell line unable to form tight junctions but used to assess toxicity as well as to study epithelial structures⁹¹. On the other hand, alveolar epithelial cell lines are employed to resemble the alveolar epithelium which is formed by alveolar type 1 and 2 cells. A549 and NCI-H441 cells are the most common alveolar cell lines used in research⁹². Due to their immortalised origin and consequent tumorigenic potential, the use of cell lines is restricted to *in vitro* models and *in vitro* characterisation of potential airway substitutes.

In comparison to cell lines, primary airway cells have limited availability, require complex subculturing protocols and may suffer from de-differentiation and donor variability¹⁶. However, they can better mimic the respiratory epithelium as primary cells have not been modified to increase proliferation and have been reported to differentiate into all cell types found in the airway epithelium⁹³. Normal human bronchoepithelial (NHBE) cells are the most used primary tracheobronchial cell and can be incorporated into respiratory *in vitro* models in order to minimise the use of animal models and better represent lung mechanisms⁹⁴. Primary bronchial epithelial cells (HBEs) have also been employed during epithelialisation strategies of TE tracheal grafts^{84, 95, 96}. Nasal epithelial cells have also been investigated as a primary cell population for the regeneration of the tracheobronchial epithelium⁹⁷. Primary airway cells can also be used for the creation of advanced *in vitro* models and offer

the possibility to develop disease specific models such as CF and asthma^{98, 99}. Extensive research has also been carried out in the field of stem cells for respiratory tissue engineering applications. Airway progenitor cells can be obtained directly from the respiratory tract but can also be sourced exogenously from other tissues such as embryonic stem cells (ES), induced pluripotent stem cells (iPSC) or amniotic fluid stem cells (AFSC)¹⁰⁰.

The trachea not only comprises a respiratory epithelium as explained in Section 1.2.1.1, but also comprises a wide variety of tissues of crucial importance when developing airway *in vitro* models through co-culture strategies as well as for the development of airway grafts for tissue regeneration. As hyaline cartilage is one of the main components of native trachea, chondrocytes have been widespread used as a cell source for tracheal regeneration. Chondrocytes can be directly isolated from auricular, nasal septum, rib and tracheal cartilage¹⁰¹. For example, human tracheal chondrocytes have been harvested from patients in need of tracheal replacement and seeded into biodegradable scaffolds. They led to the development of new cartilage tissue with characteristics that reassemble native tracheal cartilage¹⁰². Nasoseptal chondrocytes can also represent an alternative cell source as it has been shown that sheep-derived nasal chondrocytes developed tissue with suitable mechanical properties and glycosaminoglycan content comparable to the one of native trachea. Interestingly, isolation of these nasoseptal chondrocytes is less difficult and invasive than the technique used to obtain tracheal chondrocytes⁷³. Nonetheless, primary chondrocytes have limited expandability *in vitro* and commonly suffer from de-differentiation¹⁰³. In order to overcome these limitations, research efforts have moved towards the use of stem cells such as adult mesenchymal stem cells (MSCs). MSCs can be isolated from bone marrow or adipose tissue and be differentiated into mature chondrocytes by a wide range of paracrine soluble chondrogenic growth factors, mechanotransduction signalling pathways and the use of co-culture models¹⁰¹. iPSCs are also a promising cell source for tracheal chondrocytes as they avoid ethical concerns and immune rejection by the recipient. For instance, iPSCs were seeded onto collagen-based 3D scaffolds to achieve chondrocyte differentiation *in vitro* and later on implanted into tracheal defects of nude rats. The scaffolds were demonstrated to promote the formation of tracheal cartilage in the injury site making it a promising alternative cell source for tracheal regeneration¹⁰⁴. Other cell types of interest in

tracheal tissue engineering are human umbilical vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HMVEC-Ls) to promote vascularisation in tracheal tissues¹⁰⁵. Immune cells have also been applied in synthetic tracheal tissues to evaluate the epithelial response to inhaled substances and have been studied in tracheal graft prototypes^{95, 106}. The use of immune cells in tracheal tissue engineering applications is further expanded in Section 1.4.

Table 1. 2. Airway epithelial cell populations available for respiratory and trachea tissue engineering.

Cell Population	Classification	Application	References
Calu-3	Cell Line	– <i>In vitro</i> and Disease Modelling – Drug Delivery, Deposition, and Transport – Aerosol Deposition – Bacterial Invasiveness – Pulmonary Drug Disposition	88, 107
16HBE14o–	Cell Line	– Epithelial Diseases Modelling (CF, COPD, Asthma) – Barrier Function Modelling – Drug Delivery, Transport and Development	89, 90, 108
BEAS-2B	Cell Line	– <i>In Vitro</i> and Disease Modelling (Lung Carcinogenesis) – Toxicology Testing	109, 110
NHBEs	Primary Cells	– <i>In Vitro</i> Modelling – Therapeutics Development – Disease Modelling (CF, viral infection) – Airway Biology	94, 111, 112
HBECs	Primary Cells	– Airway Biology – Disease Modelling – Airway Regeneration	84, 113, 114

Nasal Epithelial Cells	Primary Cells	– Disease Modelling (Inflammation, Viral Infection) – Airway Biology and Stem Cell Research – Airway Regeneration – Drug Development and Absorption	115-119
ES	Progenitor Cells	– Cell therapy of injured epithelium – Disease modelling – Airway regeneration – Airway biology – Drug development	120-124
iPSC	Progenitor Cells		
AFSCs	Progenitor Cells		

1.3.2 Biomaterials for Respiratory and Trachea Tissue Engineering

Both TE *in vitro* models of the tracheobronchial region and approaches for tracheal regeneration rely on the use of biomaterials to provide a 3D environment –or scaffold– for cells to grow, irrespective of the final application. Generally, biomaterials are classified into natural and synthetic materials (Figure 1.3B). While natural materials comprise those involving ECM components (e.g. collagen hyaluronic acid (HyA) and alginate) and decellularised matrices, synthetic materials refers to polymers, ceramics and metals¹²⁵. Both natural and synthetic materials must possess certain characteristics in order to successfully be employed as biomaterials (Figure 1.3A). These materials must be biocompatible and allow for cell attachment, adhesion and proliferation. Depending on the application intended, further characteristics may be required including tunable biodegradability, porosity and antimicrobial properties. The range of different materials available offers the possibility to tailor scaffold characteristics for specific applications including mechanical properties and porosity¹²⁶.

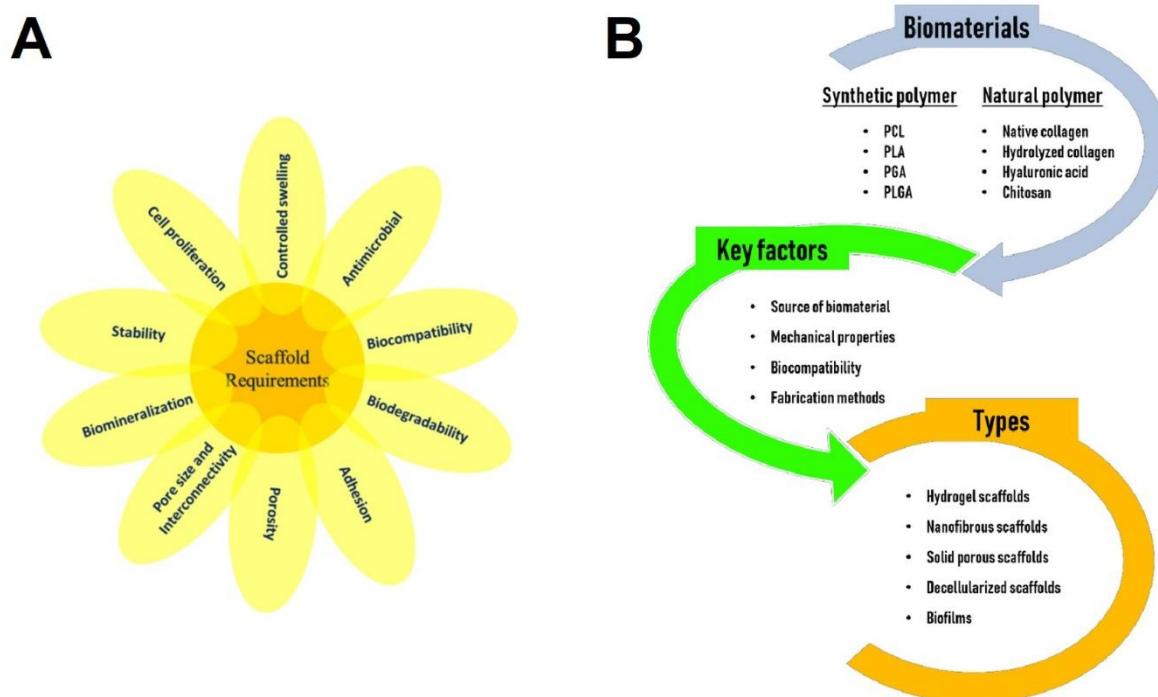


Figure 1. 3. Biomaterials for tissue engineering. (A) Characteristics and properties of synthetic and natural biomaterials for its use in tissue engineering applications. (B) Types of biomaterials and key factors used to classified biomaterials. Adapted from^{125, 127, 128}.

1.3.2.1 Natural materials

Natural polymeric materials used in tissue engineering consist of long chains of monosaccharides (e.g. chitosan, alginate and HyA) or aminoacids (e.g. collagen, fibrinogen and silk) that can sometimes contain functional biomolecules to increase biocompatibility and material's performance¹²⁸⁻¹³⁰. The main advantages of natural polymers are the low toxicity of their degradation by-products, biocompatibility and natural resemblance to the native ECM. On the other hand, microbial contamination, poor control over degradation and inadequate mechanical properties represent the main disadvantages of these biomaterials. Collagen has been widely explored to develop 3D scaffolds for airway regeneration as well as for the establishment of 3D *in vitro* models^{86, 131-134}. It is one of the most common biomaterials in use, as collagen is one of the principal components of the ECM and allows for cell binding, proliferation and differentiation¹²⁹. From the more than 20 different members of the collagen family, collagen type I has served as the backbone for the manufacture of numerous TE scaffolds as its effectiveness has resulted in clinical applications¹³⁵. Porcine-derived collagen scaffolds have been demonstrated *in vivo* to promote the regeneration of

respiratory epithelium and, in combination with heparin, have been shown to promote re-epithelialisation as well as cartilage formation¹³⁶⁻¹³⁸. HyA scaffolds seeded with human mesenchymal stem cells (hMSCs) have been used for tracheal regeneration and as *in vitro* models of respiratory tract infections^{139, 140}. Electrospinning of decellularised airway ECM has also been employed to develop *in vitro* models of the airway wall¹⁴¹. The use and application of decellularised tissue for tracheal TE grafts is further discussed in Section 1.3.3.1. Other natural biomaterials that have been applied for respiratory tissue engineering applications include chitosan^{142, 143}, gelatin¹⁴⁴⁻¹⁴⁶, fibrin¹⁴⁷ and silk¹⁴⁸.

1.3.2.2 Synthetic Polymers

Synthetic polymers offer key advantages over natural biomaterials, including tunable properties such as mechanical strength and chemical composition. For example, numerous synthetic polymers allow for tunable molecular weights and chemical features, offering easy functionality of the polymer chains versus natural polymers^{129, 149}. Moreover, the mechanical properties, degradation rates and other physical characteristics are well determined, predictable and reproducible between batches¹²⁹. However, the use of synthetic polymers for respiratory tissue engineering might be restricted due to the low capacity of these polymers to sustain cell attachment, compromised biocompatibility in some cases and possible immune response¹⁵⁰. The most common synthetic polymers used in TE are polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), poly lactic-co-glycolic acid (PLGA), poly(ethylene oxide) (PEO), polyethylene glycol (PEG) and polyurethane (PU)¹⁵¹. Several of these polymers have been investigated for the formulation of novel nanoparticles for respiratory applications and inhaled therapies¹⁵²⁻¹⁵⁴. On the other hand, several scaffolds have been manufactured using synthetic polymers with enhanced mechanical properties over their natural analogues for airway repair and regeneration¹⁵⁵. To-date, several synthetic polymers have been applied as scaffolds for tracheal regeneration including PCL, polyethylene terephthalate (PET) and PU composites, polyester-urethane and PLA¹⁵⁶⁻¹⁵⁹. The most extensively used synthetic polymer for tracheal regeneration is PCL due to its excellent biocompatibility, biodegradability and approval by the U.S. Food and Drug Administration (FDA) in a range of clinical products such as intrauterine systems, sutures, birth control devices

and spheres for drug delivery¹⁶⁰⁻¹⁶³. In order to take advantage of the versatility and tunability of synthetic materials while retaining the outstanding biocompatibility and cell attachment from natural materials, composite scaffolds have been also investigated for airway repair¹⁶⁴⁻¹⁶⁶. For example, electrospun PCL scaffolds have been combined with alginate for bone regeneration while PLGA/collagen scaffolds have been investigated for articular cartilage repair^{167, 168}.

1.3.2.3 Novel Polymers – Polyglobalide (PGL)

Although there is a wide range of different synthetic polymers, there is a need for novel materials that while carrying many advantages of synthetic polymers also include those of natural materials regarding cell attachment and growth¹⁶⁹. Interestingly, the surface of some of these polymers allow post-processing functionalisation or decoration to mimic motifs that increase cellular attachment and proliferation¹⁷⁰. An example of these polymers can be found in polyglobalide (PGL), an unsaturated macrolactone that derives from the monomer globalide (GL). Side chain functionalisation of PGL has opened the possibility for post-processing modifications and its potential for biomedical applications in comparison to widespread biomedically used polymers such as PCL and PLGA^{170, 171} (Figure 1.4). Macrolactones are aliphatic polyesters with a cyclic structure that have been described to be an alternative bioresorbable thermoplastic for tissue engineering applications, with a tunable biodegradability rate over time and the possibility of sophisticated surface modifications¹⁷⁰. The cytotoxicity of PGL has been studied by using mouse-derived fibroblasts that were cultured in the presence of different concentrations of the polymer. This study showed no toxic PGL effect on cell viability when using 0.1 g/mL of the polymer suspended in growth medium in the presence of mouse fibroblasts¹⁷². PGL-based fibrous scaffolds were also manufactured using electrospinning and a method for drug loading of the scaffolds was evaluated. PGL fibres were successfully loaded with Rhodamine B and indomethacin, an anti-inflammatory drug, highlighting the potential of PGL to be used in both tissue engineering and drug delivery applications. The PGL scaffolds were also seeded using MSCs and were able to support cell viability and proliferation¹⁷³. Finally, surface modification of PGL electrospun scaffolds with amino groups and Arg-Gly-Asp (RGD) sequences has been shown to increase the attachment and proliferation of hMSCs^{170, 173}.

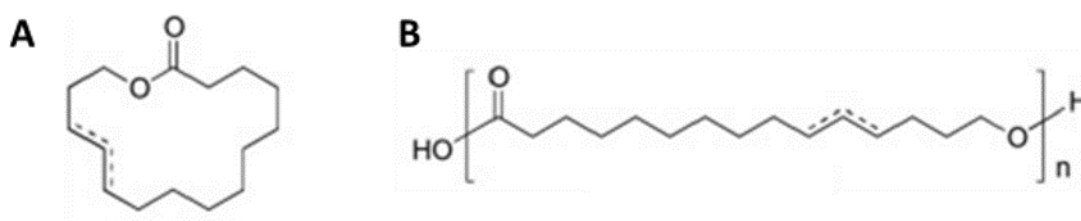


Figure 1. 4. Chemical structure of GL (A) and PGL (B). Adapted from¹⁷¹.

1.3.3 Manufacturing Techniques

Scaffolds for tissue engineering are designed to meet a specific set of criteria for their intended use. The manufacturing technique chosen depends on the final application as well as the characteristics including mechanical properties and architecture required for a specific tissue¹⁷⁴. The development of tissue-engineered trachea (TET) grafts has focused on tubular designs to mimic the hollow tube structure of the trachea while maintaining adequate porosity to support tissue growth and regeneration^{22, 35, 85, 175, 176}. The most common techniques used in airway scaffold system production are decellularisation, casting, electrospinning and 3D printing (Figure 1.5). Some techniques allow for rapid modification and design of the scaffold such as 3D printing¹⁷⁷. Scaffold architecture and porosity, which plays a key role in cell attachment and proliferation, also varies between different manufacturing techniques and can be tailored using specific techniques including electrospinning, 3D printing and freeze-drying^{25, 178, 179}.

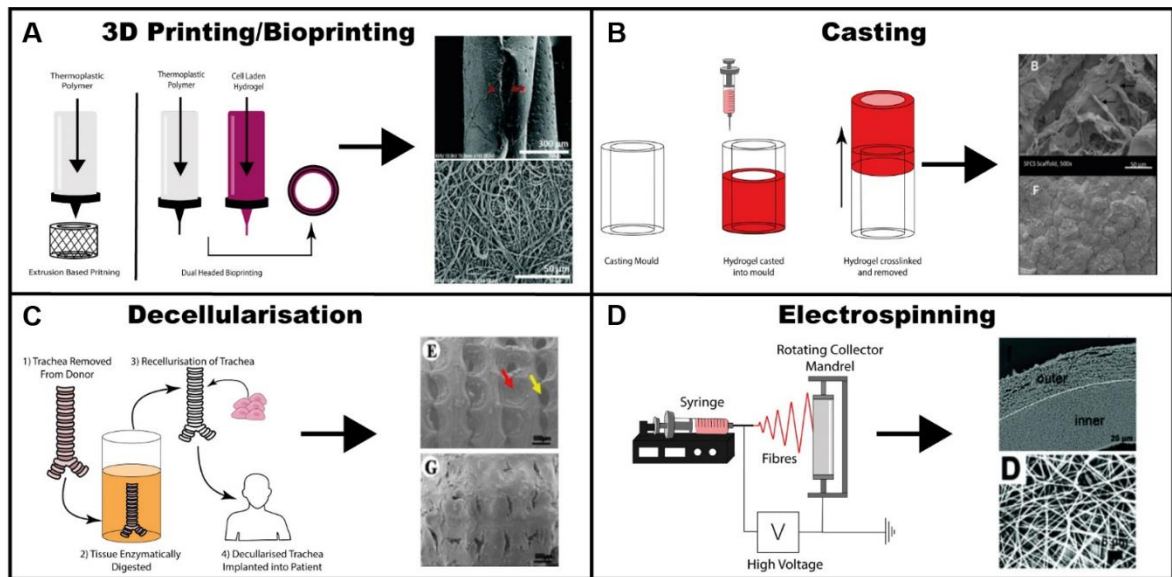


Figure 1. 5. Techniques used for the manufacture of scaffolds for respiratory and tracheal tissue engineering applications. (A) 3D printing and bioprinting, (B) casting, (C) decellularisation and (D) electrospinning. Adapted from^{176, 180-182}.

1.3.3.1 Decellularisation

Decellularisation is accomplished by subjecting tissues to repeated cycles of various detergents and enzymes over a long period of time to remove genetic material and avoid immune reactions⁷⁵. It was first developed by Baylak et al. by removing resident cells and major histocompatibility complexes for bladder tissue engineering applications¹⁸³. Decellularisation techniques allow for the maintenance of the original ECM from the native tissue and its mechanical properties so that can be used as a biomaterial for cellular growth (Figure 1.5C)¹⁷⁴. Decellularised lung scaffolds have been developed to better understand airway smooth muscle and ECM interactions, to evaluate the potential of airway stem cells and to better understand lung lineage differentiation and airway epithelial stem cells^{164, 184, 185}.

Decellularisation has also been employed for the development of tracheal grafts with promising results in revascularisation and re-epithelialisation, although graft collapse or stenosis due to an immune response has hampered translation. Complete removal of genetic material and cellular debris is not possible with existing techniques, which is likely to trigger an adverse host response *in vivo*¹⁸⁶. Complete removal would result in significant damage to the tracheal tissue, compromising its integrity as a matrix for cell growth and further weakening its mechanical properties¹⁸⁷. While decellularisation

approaches show promise by providing an established architecture for cellular growth, they are severely limited by long expensive processing methods and donor-recipient matching limitations^{75, 188}. However, the natural architecture decellularisation provides means decellularised tracheas are an excellent base for tissue growth over synthetic materials and have been combined with other TE techniques with promising initial results^{158, 188}. To avoid scarring during *in vivo* vascularisation, decellularised trachea grafts have been combined with collagen/silicone dermal regeneration templates in rabbit models¹⁸⁹. Moreover, combination of decellularised tracheas with genipin improved the mechanical strength of the graft while retaining strong angiogenic properties *in vivo*¹⁹⁰. Decellularisation stands as a promising TE technique for tracheal regeneration with success during animal *in vivo* implantation and clinical use in humans, but further research is needed to minimise scaffold damage during decellularisation assuring original ECM components are maintained and to avoid the need for stenting reinforcement months after implantation¹⁹¹.

1.3.3.2 Hydrogel Casting

Hydrogels are one of the most commonly used scaffold types for respiratory tissue engineering, allowing microenvironment engineering and stimulation of tissue regeneration. They are 3D networks of cross-linked, hydrophilic polymers that form porous environments allowing nutrient diffusion¹⁷⁴. Hydrogels allow for a degree of tunability and tailoring of their mechanical properties and degradation rates by the polymer, concentration and crosslinking method of choice^{192, 193}. Hydrogel-based 3D models of the airways have been employed to study the role of lung MSCs in pulmonary diseases, for drug screening and exploring MSC-based treatments of respiratory diseases¹⁹⁴⁻¹⁹⁶. Airway regeneration has also been explored by using collagen, hyaluronic acid, gelatin and chitosan hydrogels^{144, 197, 198}. Interestingly, the use of these hydrogels in trachea tissue engineering has also been done in combination with other techniques (e.g. 3D printing) without the need for further casting^{195, 199}. Hydrogel casting commonly employs moulds where the original polymeric hydrogel is casted and after cross-linking, a defined geometry is achieved²⁰⁰. Casting of materials has been widely used in a range of TE applications allowing better control of scaffold geometries in 3D structures and reducing variability (Figure 1.5B)⁷⁴. Casting methods in TE applications usually utilise distinctive blends

of hydrogels that are highly customisable and are able to provide a range of biochemical compositions, architectures, and mechanical properties¹⁸². Several attempts at casting of hydrogels can be found for tracheal tissue engineering. For example, tube-like scaffolds have been developed using collagen and fibroblast hydrogels and characterised both *in vitro* and *in vivo*, which were able to avoid airway collapse and maintained tracheal patency²⁰¹. Patterned 2-hydroxyethyl methacrylate hydrogels closely matched the mechanical properties of neonatal ovine tracheas while fibrin and agarose type I collagen hydrogels supported growth of culture ciliated epithelial cells and vascular networks^{74, 202}. The use of hydrogels as tracheal substitutes remains limited but initial investigations have been promising, although further research is needed to provide the required mechanical integrity and to fully interrogate the feasibility of hydrogel-based tubular constructs^{144, 199}.

1.3.3.3 Electrospinning

Electrospinning is a versatile technique that can be used with both synthetic and natural polymers, to rapidly fabricate customisable multi-layered 3D constructs using a high voltage to eject the polymer of choice onto a collector plate forming nanofibrous structures (Figure 1.5D)²⁰³. For example, electrospun PET scaffolds have been employed for the development of an upper airway scaffold for disease modelling, drug evaluation and to better model airway smooth muscle cell microenvironments^{106, 204}. Better 3D models of the airway that can be applied in airway disease research have been developed using electrospinning of and the co-culture of airway epithelial cells and fibroblasts²⁰⁵.

Electrospinning has become widely used in tracheal regeneration applications due to its ability to produce fibrous scaffolds with a similar size-scale to native respiratory ECM using PCL, PLA, PET and PU^{25, 158, 176, 206, 207}. Successful animal trials of different electrospun tracheal substitute materials have been performed, although data from a number of studies has observed a more biocompatible response in cell-loaded electrospun scaffolds than in cell-free constructs^{176, 206, 207}. Moreover, assessments of different material strength scaffolds manufactured with this technique has typically ranged from matching the human trachea to show higher mechanical strength compared to native trachea^{158, 159}. Electrospinning shows promise as a manufacturing method for TET grafts, but long-term animal studies have been limited to-date.

Electrospun scaffolds have shown to retain the architecture and porosity needed for tracheal replacement, but simplistic manufacturing methodologies to avoid complex material composites used or the combination of electrospinning with other techniques such as decellularisation and 3D printing will allow easier and faster translation of these scaffolds^{176, 208, 209}.

1.3.3.4 3D printing

Additive manufacturing, commonly referred as 3D printing, is now extensively utilised for research purposes in the medical field as it allows for rapid fabrication of custom scaffolds for tissue replacement that can be tuned to alter properties including architecture, mechanical properties and rate of degradation^{210, 211}. In comparison to the other techniques, 3D printing allows to better control scaffold's porosity, pore interconnectivity, biological and mechanical properties¹⁷⁴. 3D printed porous scaffolds seeded with human HBECs have been used to create a novel *in vitro* model of the airway and it has been applied for the creation of airway-on-a-chip models that can serve as platforms for preclinical trials in biomedical research^{212, 213}.

3D printing also facilitates the generation of complex multi-layered designs that can be personalised to meet a specific patient's tracheal anatomy. It is therefore commonly used in regenerative therapies to enable rapid prototyping of a range of designs with tunable mechanical properties²¹¹. The most commonly explored material in 3D printing for TE applications has been PCL due to its excellent mechanical properties, long-term stability and slow *in vivo* degradation rate. Recently, 3D printed external tracheal splints made from PCL were granted FDA approval for emergency use in paediatric surgery to correct tracheobronchomalacia (TBM) successfully²¹⁴. 3D airway splinting differs from TE scaffolds as they are used as standalone materials for airway support. Although tissue growth is achieved, the 3D printed implanted splint just acts as support to guide airway growth and avoid collapse until airway maturity is achieved in the patients. However, it is important to note that 3D printed PCL scaffolds have had very little success in animal studies due to an inflammatory response, granulation and stenosis²¹⁵. Other materials have also been investigated as 3D printable materials for TET grafts including PU and polyether ether ketone (PEEK)^{175, 216}. Attempts at 3DP of tracheal scaffolds with various materials to create unique tubular designs are still ongoing and have shown promising initial results but studies have been limited by

single cell type approaches, poor mechanical characterisation and insufficient re-vascularisation²¹⁷. Bioprinting, a type of 3D printing based on direct cell-laden biomaterial printing has also been investigated for tracheal regeneration but constructs to-date have demonstrated inadequate mechanical strength²¹⁸. To overcome this, combinations of synthetic polymer-based 3D printed scaffolds with hydrogels such as coating with collagen type I and 3D printing alternating PCL layers with alginate hydrogels containing MSCs have also been explored^{219, 220}. 3D printing has shown promising *in vitro* and *in vivo* experimentation while demonstrating translation with some approaches used in clinics. However, future work should investigate the use of standalone polymers to avoid combination with natural polymers while the ability of 3D printed TET scaffolds should be carefully study to support epithelialisation and vascularisation ^{214, 220-222216, 222-224}.

1.4 In Vitro Models of the Tracheobronchial Region for Drug Development

In vitro models of the airway, and more specifically of the tracheobronchial region, should closely mimic the relevant airway epithelial barrier. Effective models may be applied in pre-clinical drug development, non-clinical safety and toxicological testing²²³. Moreover, they provide platforms to better understand the pathophysiology of specific disease models offering a promising platform to better understand respiratory diseases and develop novel treatments^{224, 225}. *In vitro* airway models have advanced in complexity over the last decades e.g. the formation of a mature epithelium under ALI conditions or establishment of complex 3D co-culture systems (Figure 1.6)⁸⁶. ALI cell based models allow to recapitulate the airflow of the respiratory system and mimic the *in vivo* respiratory environment by forming tight junctions and secreting mucus. They provide an important platform for assessing drug deposition, transport, efficacy and cytotoxicity. However, *in vitro* ALI models require further complexity to better represent the physiological and anatomical characteristics of the airway²²⁶. To overcome the limitations of the current models, tissue engineering, microfluidic and organ-on-a-chip technologies are being explored. They allow for the creation of biomimetic tissue in which epithelial cells can be grown under ALI conditions in the presence of other relevant cell types and/or in 3D environments mimicking the ECM. Furthermore, the use of these models reinforces the use of the concept of reduce, refine, or replace -3R approach- of animal testing for respiratory medicine and disease modelling²²⁷.

During the last decades, cell line-based *in vitro* models have been extremely useful for the study of respiratory diseases as well as for the development and testing of numerous compounds and formulations²²⁸. It has been proposed that the culture of cells on two dimensional (2D) plastic plates does not fully represent the complex microenvironments present in the airways²²³. Therefore, these models of the airway relying on the use of airway epithelial cells such as Calu-3 cells and NHBEs are grown on 2D filter inserts (e.g. Transwell® inserts) that allow formation an *in vitro* mature epithelium with characteristics that resemble those of the *in vivo* epithelium^{94, 99, 229, 230}. For example, several commercially available models of the airway are available including MucilAir™ and SmallAir™. These models allow for the formation of some respiratory epithelium characteristics such as the formation of tight junctions or mucus secretion. They are commonly grown on top of thick layers of ECM-derived

components or fibroblast feeder layers intended to better mimic the epithelium. However, they cannot be considered 3D scaffold-based models, as cell movement and interaction in a 3D environment resembling the physiological conditions of the tracheobronchial region is not supported²³¹. By including a 3D environment for cells to grow in, co-culture conditions with other relevant cell types found in the airway and switching, when appropriate, to the use of primary cells, the tracheobronchial epithelium can be better modelled^{228, 232}. It is believed that by replicating the ECM environment cell-ECM interactions within the 3D scaffold environment establish a 3D communication network that maintains tissue homeostasis and supports cell proliferation, migration and apoptosis^{233, 234}. The inclusion of relevant 3D models provides cell support and allow for maintenance of the cellular microenvironment, a pivotal role played by the ECM²³⁵. Incorporation of a 3D matrix in airway *in vitro* models allows to represent integrin ligation, cell contraction and intracellular signalling in epithelial cells that 2D models fail to recapitulate^{236, 237}. Moreover, in order to better mimic epithelial *in vivo* tissue structures, cues secreted and effector molecules stored in the ECM are required²³⁷. The composition of the ECM (e.g. collagens and proteoglycans) is involved in regulating cellular adhesion, migration and the transmission of external forces to the cell. For example, while collagen fibres of the ECM resist tensional forces, the content of proteoglycans is linked to the tissue resistance against compression^{238, 239}. Inclusion of such 3D scaffolding systems *in vitro* allows to better resemble native epithelial tissue architecture consisting of a polarised epithelium located on a thin basement membrane and a sublayer rich in ECM components harbouring other relevant cell types such as fibroblasts²³⁷.

Several TE models of the airway have been developed so far with improved features of the airway that are not present in conventional 2D models –filtered culture platforms such as Transwell® inserts^{240, 241}. 3D models of the airway have been employed to increase understanding of epithelial responses to allergens and foreign particles²⁴². As an example, a novel platform was developed consisting of primary fibroblasts and NHBEs grown on Alvetex® scaffolds, porous polystyrene scaffolds which allow fibroblast to secrete proteins mimicking the ECM. This model has been shown to closely match the mucociliary differentiation of the epithelial sheet and the formation of an underlying basement membrane *in vivo* and has been applied for research into respiratory infections²²⁸.

The most critical feature of any *in vitro* cell model is their biological component; and the main cell population used across tracheobronchial models are airway epithelial cells. Whether the model is based on cell lines (e.g. Calu-3 cells) or primary cells (e.g. NHBEs), it is widely accepted that these cells must be grown under ALI conditions so that the polarised phenotype of the respiratory epithelium is properly represented¹⁹. In addition, airway epithelial cells can be grown in co-culture conditions to enhance the performance and reliability of the model, increasing its complexity. Several cell types have been co-cultured with airway cells for *in vitro* model development including immune cells (macrophages, dendritic (DC) and mast cells), endothelial cells and mesenchymal cells^{243, 244}. For example, addition of macrophages and DCs to a polarised epithelium consisting of NHBEs resulted in greater sensibility of the model for the detection of inhaled fungal pathogens, showing promise for airborne infections research²⁴⁵. The combination of epithelial cells with fibroblasts and DCs resulted in quicker maturation of a respiratory epithelium that allowed for better understanding of airway cell crosstalk after exposure to external stimuli¹⁰⁶. Furthermore, inclusion of MSCs in these models has been exploited to further understand mechanisms behind airway epithelium repair and regeneration²²⁸. Although the development of 3D models is more labour intense and more expensive than 2D platforms, they allow for modelling of an increased level of complexity resulting in data more relevant to the human *in vivo* conditions²⁴⁶. However, accessibility and simple culture conditions of commercially available filtered platforms hinders further use of complex 3D co-culture models²⁴⁷.

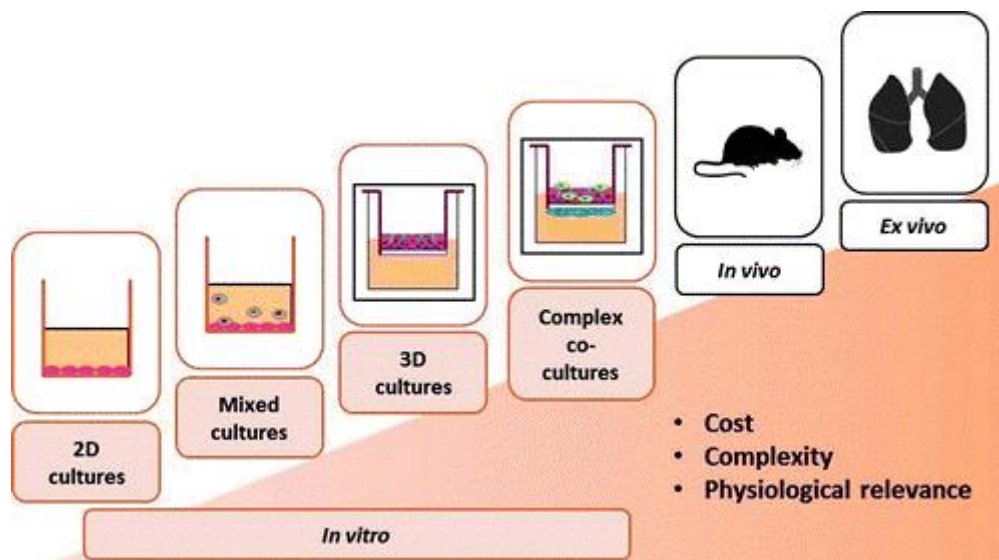


Figure 1. 6. Models of the respiratory epithelium available. These *in vitro* models range from the cheap and high-throughput 2D and can increase in complexity and physiological relevance by addition of 3D and co-culture conditions to match physiological conditions similar to *in vivo* and *ex vivo* respiratory models. Adapted from ⁴.

The slow development of novel treatments for respiratory diseases can be traced back to a range of issues including the lack of robust pre-clinical testing models, drug formulation difficulties, lack of funding and poor respiratory disease understanding¹³. Many new drug candidates fail in the clinic after showing potential in animal models, which highlights the need for more robust and accurate predictive *in vitro* models of the human respiratory tract¹⁵. The use of such models allows for exposure on respiratory epithelial cells of human origin, one advantage over the poor translation between *in vivo* animal modelling and human physiology²⁴⁸. Moreover, they provide crucial insight into the uptake, transport and interaction with the respiratory epithelium while allowing for high-throughput analysis of the purposed pharmaceutical²⁴⁹. Therefore, several advanced *in vitro* models have been applied as platforms for the development of novel inhaled therapies for respiratory diseases.

TE airway organoids represent the 3D respiratory epithelium microenvironment by combining primary and progenitor lung cell populations with ECM proteins. Although organoids do not allow for the inclusion of ALI conditions, they comprise multiple cell types that resemble the architecture of the airways²⁵⁰. These respiratory organoids can be used in different set-ups as a valuable *in vitro* drug screening platform. For example, drug microinjection into the lumen of the organoids has been used to determine the therapeutics' efficacy and cytotoxicity²²⁶. Moreover, organoids can be

embedded in microfluidic devices, which expose the airway cells to the preclinical therapeutic. Jung et al. used a microfluidic-based lung cancer organoid culture platform to mimic the drug uptake of cisplatin and etoposide, chemotherapeutics used in a small lung cancer model²⁵¹. Tissue engineering has also enabled the development of complex 3D *in vitro* models with polarised epithelial cells that can be used for preclinical drug assessment²⁵². For example, the effect of nanoparticle exposure using multi-walled carbon nanotubes (MWCNTs) and asbestos fibres on cell-matrix interactions has been studied using a 3D lung microtissue model containing epithelial cells, fibroblasts and macrophages. Exposure using this 3D models was able to predict chronic pulmonary endpoints and acute inflammation via increases of gene and protein expression such as matrix metalloproteinases (MMP1 and MMP3) and pro-inflammatory cytokines (interleukin-1-beta (IL-1 β), interleukin 6 (IL-6) and tumour necrosis factor-alpha (TNF- α), creating an alternative advance *in vitro* platform for drug toxicity testing²⁵³.

The use of 2D cell-based epithelial models has proven to be an outdated platform with restricted benefits for drug delivery and development as evidenced by poor *in vitro in vivo* correlations²⁵⁷. Although some *in vitro* platforms with a higher degree of complexity have been developed and closely mimic the respiratory epithelium, there is still a need for the translation of these TE *in vitro* models into functional platforms for drug screening^{242, 258}. Moreover, better models that can be integrated with high-throughput drug screening approaches to systematically assess drug candidates are needed. The development of state-of-the-art airway *in vitro* platforms has the potential to allow for cheaper and effective drug development²⁵⁵.

1.5 Tissue-Engineered Approaches for Tracheal Regeneration

Respiratory tissue engineering also focuses on regenerative strategies for airway repair, mainly tracheal restoration. Tracheal damage is commonly associated with the narrowing, weakening or discontinuity of this portion of the airway and is often a life-threatening condition³⁵. Defects in the conducting portion of the lower respiratory tract arise from a wide variety of pathologies. While tracheal dysfunction in adults is mainly caused by traumatic injury, cancer and TBM, congenital disorders such as tracheoesophageal fistulas, oesophageal atresia and other birth abnormalities are responsible for tracheal dysfunction in infants²⁵⁴⁻²⁵⁹. Finally, intensive care unit patients with prolonged intubation may develop laryngotracheal injuries due to endotracheal intubation procedures and tracheotomies. The incidence of post-intubation tracheal stenosis has been estimated in 6-21% of patients that underwent intubation^{260, 261}. Moreover, recent studies suggest that around 5% of COVID-19 patients and 30% of hospitalised cases during 2020 required invasive mechanical ventilation and could develop tracheal stenosis once extubated^{262, 263}. Currently, short length tracheal damage is typically corrected by thoracic surgery including tracheal resection and end-to-end anastomosis, although this is not advised for those defects exceeding 2 cm in children and 5 cm in adult patients²¹⁷. In such cases, patients are treated using slide tracheoplasty, tracheal reconstruction, and novel artificial prostheses. Several complications, however, including granulation tissue formation and weakening of the implant over time, critically endanger their performance^{35, 264}. Therefore, there is an urgent need to develop novel solutions to promote the regeneration of native tracheal tissues²⁶⁵.

In order to develop new implants as tracheal substitutes that replicate physiological form and function, the complex biological and anatomical characteristics of the trachea outlined in Section 1.2.1 and 1.2.2 must be understood, including cellular function, mechanical properties, and vascularisation. First of all, any proposed construct must be airtight and hold the potential to regenerate pseudostratified respiratory epithelium to prevent bacterial infection²⁶⁶. Secondly, a scaffold for tracheal replacement must demonstrate lateral rigidity and longitudinal flexibility to withstand inspiratory and expiratory forces, so that stent fractures due to excessive coughing are avoided²⁶⁴. Moreover, it should resist forces arising from coughing, neck movements and the pressure created by the adjacent oesophagus during feeding³⁵. Mismatched

properties will inevitably result in graft failure and airway collapse²⁰⁶. Finally, vascularisation of the implant is a key factor that has hampered the success of tracheal grafts to-date. It is of critical consideration to avoid tissue necrosis in the implant site and to support new cartilage and epithelium formation²⁶⁷. In summary, due to the high complexity of the tracheobronchial region, any tubular approach aimed to regenerate tracheal tissues must offer a suitable 3D environment for the formation of a respiratory epithelium and hyaline cartilage while allowing the development of a vascularised plexus to avoid necrotic tissue formation. Moreover, it should allow the infiltration of immune cells from the host immune system to avoid infection by inhaled pathogens during the regeneration of the respiratory epithelium.

1.5.1 Early Attempts at Tracheal Regeneration and Restoration

Early efforts for long-segmental tracheal defects focused on reconstructing the trachea with the use of polymer prosthesis in either solid or porous form. The Neville prosthesis, a solid siloxane-based prosthesis, demonstrated success in animal studies but led to stenosis and graft failure in humans due to mismatched mechanical properties^{72, 268-270}. Other solid prostheses have consisted of stainless steel wire²⁷⁶, polyethylene (PE)²⁷¹, tantalum²⁷² and silicone coupled with a Dacron™ ring²⁶⁹. However, the inability to fully restore tracheal patency turned researchers towards porous prosthesis to allow for better ingrowth of cells and tissue formation. For example, Marlex™ mesh prosthesis, provided 16 months' graft survival and patency in an animal study but failed in humans due to stenosis and erosion of surrounding blood vessels²⁷⁰. Other porous prostheses have included wire-enforced dermal grafts²⁷³, stainless steel wire mesh²⁷⁴, silicone with Dacron™²⁶⁸, PE²⁷⁵ and silicone tubes²⁷⁶; but these have had similar poor results. Current treatment options for tracheobronchial complications are based on the use of commercially available stents including the Ultraflex™²⁷⁷, silicone-based self-expanding PolyFlex™ from Boston Scientific²⁷⁸, and Novatech's silicone Dunmon® and Gold Studded Stents®²⁷⁹. The use of silicone stents has been shown to improve symptoms and quality of life in the vast majority of clinical cases, although long-term follow up studies have highlighted serious drawbacks such as stent migration, the formation of granulation tissue, obstruction, and mucus retention with infections²⁸⁰⁻²⁸³.

Allograft transplantation for large tracheal defects has primarily focused on two donor tissue sites: tracheas and aortas. Fresh and cryopreserved aortas have been shown to regenerate cartilage and respiratory epithelium in an ovine model, albeit cartilage regeneration in other animal studies has not been observed²⁸⁴⁻²⁸⁶. Several studies have seen the implantation of aortic allografts in humans with poor results and serious complications including blood vessel erosion, stent migration and tracheoesophageal fistulas, with no cartilage formation or respiratory epithelium observed in biopsied tissue²⁸⁶⁻²⁸⁹. The most extensive trial using aortic allografts involved 20 patients with a 76.9% survival after airway transplantation with regeneration of respiratory epithelium and cartilage formation. However, authors highlighted the need to further assess both the efficacy and safety of this procedure²⁹⁰. Following the early successes using tracheal allografts, attempts at trachea transplantation have seen very little clinical success^{72, 291-293}. Recent studies saw the development of a two-step technique in which the donor trachea is revascularised in the patients forearm before implantation²⁹⁴⁻²⁹⁷. Despite the tissue necrosis that was observed in some patients, the technique remains a promising approach^{72, 296, 297}. However, it is not suitable for patients with malignant tumours due to the need of immunosuppressive therapy and allografts need to be supported with stents due to inadequate mechanical properties and cartilage regeneration^{72, 264}. Revascularisation of allografts is poor resulting in the need for vascularisation by implantation into a second surgical site, increasing the risk of infection and hospitalisation time^{294, 297}.

1.5.2 Pre-clinical Outcomes of Tracheal Tissue Engineering Approaches

Based on the limitations on the previous attempts for tracheal regeneration, tissue engineering offers a promising alternative for the treatment of long segmental defects^{22, 298}. Translation of TET into animal studies has been performed with some limited success. Many of these attempts are difficult to interpret given the limited follow up time of the treated animals, around 1-3 months in most cases^{299, 300}. Early attempts often ended in a severe inflammatory response leading to granulation, stenosis or graft failure through infection although pre-implantation strategies to promote vascularisation increased survival³⁰¹⁻³⁰⁴. Wrapping the construct in omentum provides both a vascular network and a source of tissue to seal and maintain airtightness of the graft, thus creating a barrier to bacterial colonisation. Pre-implantation in the abdominal cavity facilitates omentum adherence to the tracheal graft and integration

of the vascular network³⁰⁴. Pre-vascularisation of electrospun and bioprinted TET scaffolds prior to implantation has shown higher survival rate of animals, lower rates of stenosis and increased re-epithelisation (Figure 1.7A)^{176, 220}. Construct pre-seeding with chondrocytes and pre-vascularisation has also been reported to increased survival rate of animals improving tissue regeneration¹⁷⁶. Moreover, Bae et al. demonstrated that the inclusion of pre-seeded epithelial cells into a PCL-based 3D bioprinted scaffold enhanced graft acceptance and overall implant success in conjunction with MSCs, resulting in full epithelial coverage of the graft and no signs of distress or graft failure²²⁰. The longest running preclinical study to date was performed in a canine model with a 24 month follow up using a collagen coated nitinol frame wrapped in omentum. Histological examination showed stable epithelialisation in a non-stratified monolayer with no secretory glands present and no muscular regeneration but stenosis was not observed (Figure 1.7B)³⁰⁴. Other studies using this approach in which porous PLGA scaffolds pre-seeded with epithelial cells have also observed similar results after 2 months, unfortunately longer term follow ups were not published³⁰⁹. Several other studies in canines involving epithelial and collagen covered polypropylene (PP) meshes have been followed up to 5-12 months^{305, 306}. As previously highlighted, the lack of functional epithelium resulted in significant risk of infection, inflammation and/or graft encapsulation. Ongoing inflammation was present in a study using composite polyethylene terephthalate (PETE) and PU implanted in sheep, leading to stenosis formation most evident at proximal and distal locations of the graft. Seeding of PETE/PU electrospun grafts with MSCs was associated with delayed onset of respiratory distress showing the importance of establishing a functional epithelium prior to implantation²⁰⁶. These *in vivo* trials of TET technologies have highlighted significant challenges that need to be addressed. The inclusion of a pre-seeded epithelial layer appears to mitigate bacterial colonisation and the pre-vascularisation of grafts also enhances graft acceptance and survival, however there are still concerns due to the presence of granulation.

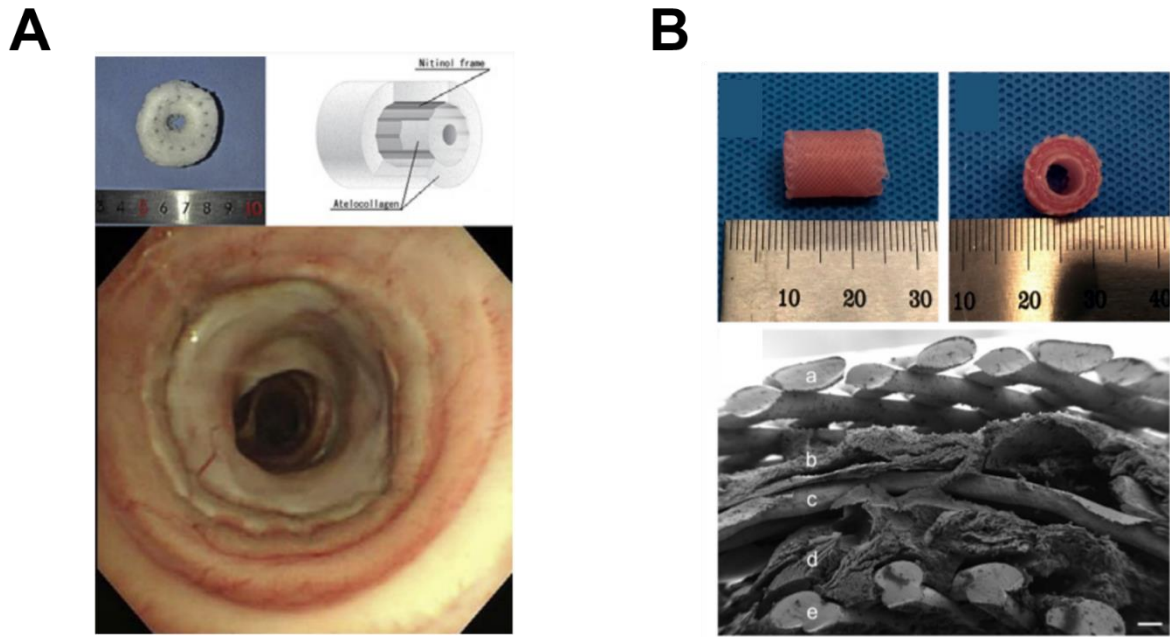


Figure 1. 7. Examples of pre-clinical prototypes of tracheal grafts used in animal models for regeneration. (A) Collagen-coated nitinol frame implanted into a canine model showing cell engraftment and survival up to 18 months. (B) 3D bioprinted tracheal scaffold containing rabbit MSCs and rabbit epithelial cells using a 3D printed cylindrical shape made of PCL. Adapted from ^{220, 304}.

1.5.3 Clinical Translation of Tissue-Engineered Tracheas

Few TE approaches for tracheal regeneration have made it to human clinical trials but significant findings have emerged from those assessed clinically to-date. For example, the implantation of several TE scaffolds which lack *in vitro* and *in vivo* characterisation lead to the death of several patients creating a scientific misconduct and ethical scandal, hindering clinical translation of TET grafts^{307, 308}. This lead to the need for better characterisation of any proposed construct during the development process while vascularisation was proven to be essential for any TET scaffold. In 2005, the first report of a successful case employed a Marlex prostheses covered in type I and II porcine-derived collagen and regeneration of the respiratory epithelium and full incorporation of the scaffold within surrounding tissues was observed²⁹⁸. This technique was further employed in a wider set of patients demonstrating a well-epithelialised lumen with no apparent airway obstruction in follow-up examinations (Figure 1.8A)³⁰⁹. Moreover, this approach has gained further complexity with the addition of basic fibroblast growth factor (bFGF) to the cartilage defect to improve blood vessel formation and was shown to maintain airway patency in all patients³¹⁰.

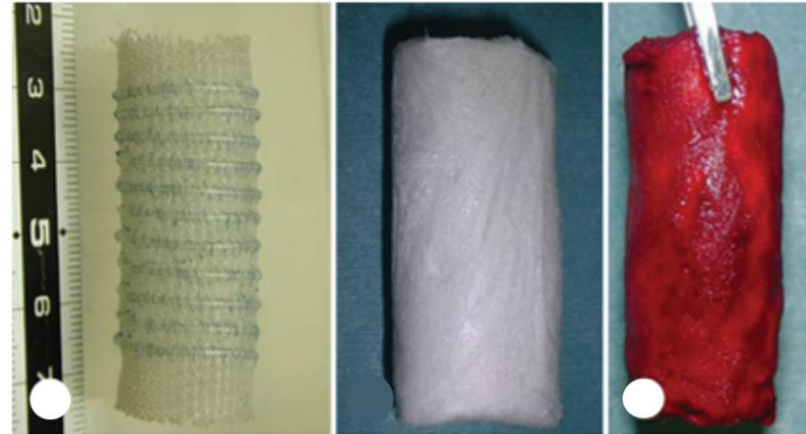
Surgical implantation of decellularised tracheas was first successfully performed in 2010. They were seeded with buccal mucosa to enhance vascularisation prior to implantation³¹¹. This approach was further explored with four additional patients, but tracheal necrosis and poor vascularisation were detected³⁰⁹. Decellularised tracheas have also been pre-seeded prior to implantation with the recipient's own stem cells and added growth factors with mixed outcomes due to graft narrowing³¹²⁻³¹⁴. Even though some of these TE approaches have demonstrated a certain degree of promise, further and more extensive follow-up studies and a wider number of patients will be needed in order to ensure this technology can become an alternative approach for tracheal restoration and regeneration. Moreover, extensive prototyping and better pre-clinical testing models are highly needed to ensure progress on clinical translation.

3D airway splinting has also been investigated in patients suffering from airway defects. The implantation of a 3D printed splint to treat a severe case of TBM was firstly reported in 2013³¹⁵, and since then has been applied to a wider set of patients reporting low general mortalities (Figure 1.8B)^{211, 214, 316, 317}. However, airway splints have been mostly restricted to paediatric patients suffering from TBM. Using this approach, airway growth is guided during the early stages of development and leads to natural resolution of TBM. The use of 3D airway splint allows for the development of personalised devices with similar mode of action of widely used airway stents. Here, airway patency is maintained to allow tissue regeneration or growth, but they are not themselves promoting the regeneration of tracheal tissues in comparison to the previous TE examples. Apart from paediatric patients, there is only one reported attempt of using a 3D printed airway splint in an adult individual with maintained airway patency three months after the procedure³¹⁷. Several patients have successfully received silicon 3D printed implants custom made from CT-scan images in clinical trials, with reports of improved quality of life and no complications³¹⁸.

The promising results of 3D printed airway splints similar to current stenting technologies could be used as the foundation and background for more elaborated 3D printed approaches that do not only maintain airway patency but are also intended to regenerate the airway tissues on the site of the defect. Albeit early promising attempts to use 3D printing technologies to overcome tracheal stenosis and malignancies, there is a need to extend follow up studies to assess splint resorption as well as establish

optimal designs and validated manufacturing processes in order to assure safety and efficacy during future clinical trials when combined with a TE regeneration approach³¹⁹.

A



B

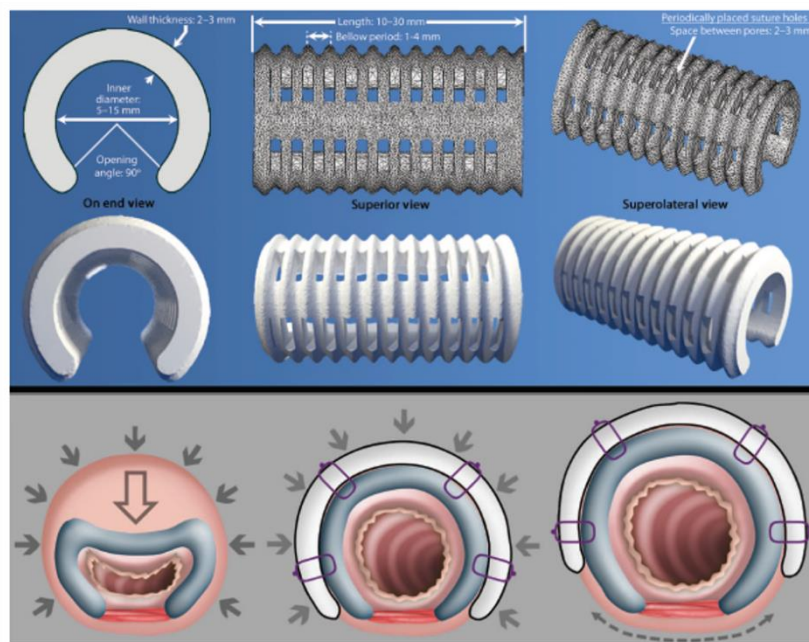


Figure 1. 8. Examples of clinical translated strategies for tracheal repair in humans. (A) Marlex mesh tube reinforced with a propylene ring and coated with porcine dermal atelocollagen that after extensive *in vitro* and *in vivo* characterisation was used in a set of 4 patients for larynx and trachea repair. Follow up studies up to 34 months post-implantation demonstrated epithelialisation of the implanted graft and no stenosis observed³⁰⁹. (B) 3D printed airway splint made of PCL for tracheal restoration. The 3D airway splint was 3D printed to develop a personalised medical device for airway collapse induced by TBM. After implantation and follow up assessment in 3 infants, no signs of TBM were detected showing a prolonged and successful airway growth²¹⁴. Images adapted from ^{214, 309}.

1.6 Thesis Objectives

The central hypothesis was that tissue engineering strategies can be used to create upper airway *in vitro* models of the respiratory tract for drug development and testing, and be further exploited as regenerative approaches for tracheal replacement and restoration. Therefore, the overall aim of this thesis was to develop improved and physiologically-relevant respiratory tissue analogues of the upper airway region that can be used in respiratory drug development and can also be harnessed as tubular constructs for tracheal regeneration.

The specific objectives of this thesis are:

- To assess collagen hyaluronic acid bilayered (CHyA-B) scaffold airway co-cultures as an *in vitro* model to recapitulate physiologically-relevant toxicological and inflammatory responses to stimuli and compare to current 2D standard models (Chapter 2).
- To harness CHyA-B airway co-culture models to assess the safety and toxicity of nanomaterials exposed to epithelial co-cultures (Chapter 3).
- To assess the utility of polyglycolide (PGL) as a novel, 3D printable biomaterial for tissue engineering applications (Chapter 4).
- To manufacture and characterise tubular tissue-engineered trachea combining 3D printed polycaprolactone (PCL) or PGL constructs with CHyA-B scaffolds for tracheal tissue engineering applications (Chapter 5).

Chapter 2

A Tissue-engineered Tracheobronchial *In Vitro* Co-culture Model that Provides Physiologically-representative Epithelial Toxicological and Inflammatory Responses

This Chapter has been published as Soriano et al., "A Tissue-Engineered Tracheobronchial *In Vitro* Co-Culture Model for Determining Epithelial Toxicological and Inflammatory Responses." *Biomedicines* 9, no. 6 (2021): 631.

2.1 Introduction

Respiratory diseases and related infections are one of the leading causes of mortality worldwide presenting a staggering economic and social burden, with estimates of four million premature annual deaths from chronic respiratory diseases³²⁰. This includes the five major respiratory conditions -acute respiratory infections, chronic obstructive pulmonary disease (COPD), asthma, tuberculosis, and lung-related cancers- which, along with significant morbidity and mortality, are believed to account for €379.6 billion in healthcare costs annually within the European Union and \$129 billion in the United States^{321, 322}. Even though the prevalence and deleterious effects of some respiratory illnesses can be avoided and/or mitigated by promoting public health care and primary prevention measures, novel treatment options are still urgently needed to decrease the burden of respiratory disease on health and society. This has become increasingly apparent during the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) global pandemic^{15, 323}. In order to develop new and better treatments, a deeper understanding of the complex pathophysiology of respiratory diseases and better tools with which to screen new therapeutics and formulations are required.

Throughout recent decades, only a small number of novel therapies have been introduced as treatment options for respiratory diseases and it is estimated that only 3% of new respiratory drugs in the development pipeline reach final market and clinical approval¹⁵. Several factors hamper the progression of novel respiratory treatments into clinical applications, including poor understanding of disease pathophysiology and the lack of appropriate *in vitro* models to assess drug efficacy and safety early in the development process. In addition, the inhaled route offers many potential advantages for targeted treatment of respiratory diseases. It is crucial to determine efficient and safe concentrations of drugs delivered via the inhaled route that presents challenges due to the complex interplay of several pharmacokinetic processes taking place in the airways³²⁴. The complexity of the respiratory system such as the presence of multiple cell types provides many challenges to fully evaluate the impact of novel therapies and delivery systems in the intricate network of tissues involved in respiratory malfunction, which can be appreciated in the failure of many drugs candidates after *in vivo* testing³²⁵. Apart from ethical concerns regarding animal testing, it has been demonstrated that *in vivo* animal models might produce contradictory results and have poor value for translation into humans³²⁶. Moreover, the majority of pre-clinical *in vitro*

models for drug assessment fail to recapitulate the complex physiology of the upper respiratory tract by not including relevant airway cell types as well as by not representing the three-dimensional (3D) environment in which respiratory tissues are located. The widespread use of two-dimensional (2D) cultures on plastic surfaces and filter cultures, which do not resemble *in vivo* microenvironments, are described as a fundamental aspect of poor drug discovery outcomes³²⁷. The inclusion of 3D *in vitro* models could increase translation of experimental therapeutics by offering a more complex 3D environment that better mimics the extracellular matrix (ECM) surrounding respiratory tissues together with the inclusion of co-culture strategies. Therefore, there is an urgent need to develop more accurate and complex 3D models of the airways to be used as tools during drug discovery to augment clinical translation of novel therapies for respiratory medicine.

Development of advanced 3D models of the airways that could potentially enable the rapid testing of cellular responses to new therapeutics, delivery systems and even pathogens, are urgently needed. Recently, several tissue-engineered (TE) strategies have been developed consisting of biocompatible scaffolds with airway cells to better represent the native tissues of the airway^{16, 25}. The supporting scaffolds allow cells to deposit ECM components creating a relevant 3D environment, which in combination with relevant co-culturing strategies e.g. fibroblasts and bronchial epithelial cells, allows for better understanding of epithelial responses of the human airway mucosa to foreign stimuli, and/or inhaled formulations²²⁸. Our research group has recently developed an innovative collagen and hyaluronic acid bilayered (CHyA-B) scaffold that can support the formation of a submucosal tissue analogue of the conducting section of the lower respiratory tract. This model demonstrated an improved tracheobronchial phenotype over standard 2D monocultures and offers significant potential to study cellular responses in respiratory drug discovery and delivery¹³².

2.1.1 Aims and Objectives

The primary aim of this Chapter was to harness this innovative bilayered scaffold to evaluate airway cell responses following bacterial and drug-mediated challenges. The scaffold was seeded with Calu-3 bronchial epithelial cells and Wi38 lung derived fibroblasts in co-culture under air–liquid interface (ALI) conditions, followed by analysis of biological response to several stresses and comparison to conventional 2D insert

models. Overall, this Chapter sought to determine if the TE model recapitulates physiologically-relevant toxicological and inflammatory responses to stimuli better than the current 2D standard.

The specific objectives were:

- To optimise exposure parameters for bacterial and drug-mediated challenge in polarised epithelial cells grown on 2D inserts.
- To evaluate differences in toxicological and inflammatory responses of airway epithelial cells grown in 2D and 3D environments following bacterial challenge using lipopolysaccharide (LPS).
- To evaluate differences in toxicological and inflammatory responses of airway epithelial cells grown in 2D and 3D environments following drug-mediated challenge using bleomycin.

2.2 Materials and Methods

2.2.1 Materials

Unless otherwise specified in the text, all materials and reagents were supplied by Sigma-Aldrich (Arklow, Ireland).

2.2.2 Scaffold Fabrication

2.2.2.1. Collagen and Hyaluronic Acid (CHyA) Film Fabrication

Collagen and hyaluronic Acid (CHyA) films were fabricated using a modification of a previously described method^{132, 328}. A suspension of 0.5% w/v type I bovine tendon collagen (Collagen Solution, Glasgow, UK) and 0.044% w/v hyaluronic acid sodium salt from *Streptococcus equi* in 0.5 M acetic acid (AcOH) (Fisher Scientific, Ballycoolen Ireland) was blended at 4°C and 15,000 rpm for 3.5 h using an Ultra Turrax T18 Overhead blender (IKA Works Inc., Wilmington, NC). The CHyA slurry was degassed under a vacuum to remove air bubbles created during the homogenisation process; it was pipetted onto a 6x6 cm² polytetrafluoroethylene (PTFE) plate and left for 48 h under an air flow in a fume hood to produce a thin and transparent CHyA copolymer film.

2.2.2.2. Collagen and Hyaluronic Acid-bilayered (CHyA-B) Scaffold Fabrication

CHyA-B scaffolds were manufactured by freeze-drying CHyA films combined with CHyA slurry using a customised metal mould as previously described^{132, 329}. CHyA films were rehydrated for 2 h in 0.5 M AcOH, cut into 6 cm x 6 cm strips and placed onto the base of a stainless-steel grade 304 SS pan. 4 ml of CHyA slurry was pipetted on top of the film and the slurry-film combination was freeze-dried using a customised anneal cycle that initially froze samples to -20°C before heating to -10°C and holding for 24 h prior to sublimation³³⁰. After freeze-drying, CHyA-B scaffolds were cross-linked using a dehydrothermal (DHT) process at 105°C during 24 h at 50 mTorr (VacuCell 22, MMM, Planegg, Germany)³³¹.

2.2.2.3. Chemical Cross-linking

CHyA-B scaffolds were cut into discs with a diameter of 15.6 mm, hydrated in Dulbecco's Phosphate Buffer Solution (DPBS) for 30 min and chemically cross-linked

using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC). Briefly, each scaffold was cross-linked for 2 h under UV light using 2 ml of EDAC solution which consisted of 6 mM EDAC per gram of scaffold and N-Hydroxysuccinimide (NHS) in a 5:2 molar ratio of EDAC:NHS^{330, 332}. Scaffolds were washed with DPBS to remove any residual toxic product (EDAC and urea) and stored in DPBS at 4°C prior to use.

2.2.3 Cell Culture

2.2.3.1 Cell Selection and Culture Media

The Calu-3 bronchial epithelium cell line (ATCC, Middlesex, UK) was cultured in T175 tissue culture flasks (Sarstedt, Leicester, UK) following revival from liquid nitrogen storage. Calu-3 cells were seeded at a density of 1×10^6 cells per flask and were passage when they reached confluency using 0.25% Trypsin-EDTA solution. Calu-3 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% v/v foetal bovine serum (FBS) (Biosera, Ringmer, UK), 2 mM L-glutamine, 14 mM sodium bicarbonate and 100 U/ml penicillin/streptomycin; this was referred to as Calu-3 medium. Cells were used between passages 20-38. The Wi38 human embryonic lung fibroblast cell line (ATCC) was cultured in T175 tissue culture flasks following revival. Wi38 cells were seeded at a density of 1×10^6 cells per flask and were passaged when they reached confluency using 0.25% Trypsin-EDTA solution. Wi38 cells were cultured in Eagle's minimal essential medium supplemented with 10% v/v FBS, 2 mM L-glutamine, 26 mM sodium bicarbonate, 100 U/ml penicillin/streptomycin and 1 mM sodium pyruvate; this was referred to as Wi38 medium. Cells were used between passages 18-24. Co-culture medium used was a 1:1 mixture of Calu-3:Wi38 medium. Unless otherwise stated, all cell culture and incubation steps were performed at 37°C and 5% CO₂ in a humidified atmosphere.

2.2.3.2 Non-polarised Epithelial Culture

Calu-3 cells were seeded into a 12 well Corning® Costar® cell culture plate (Fisher Scientific) with 5×10^5 cells per well and grown for 48 h before toxicant exposure. Briefly, each well was pre-warmed with 500 µl of Calu-3 medium for 30 min at 37°C. Each well was then seeded using 1 ml of medium containing 5×10^5 cells and grown for 48 h prior to toxicant exposure.

2.2.3.3 Polarised Epithelial Culture on Transwell® Inserts

For epithelial monocultures, Calu-3 epithelial cells were seeded into the apical side of 12 mm Transwell® inserts (VWR, Dublin, Ireland) as shown in Figure 2.1A. Briefly, 1 ml of co-culture medium was transferred to the basolateral compartment and 400 µl into the apical compartment of the insert. The apical compartment of the Transwell® insert was then seeded with 5.65×10^5 Calu-3 cells in 100 µl of Calu-3 medium and incubated for three days. At day three of culture, medium from the apical compartment was removed to introduce ALI conditions. Cells grown on Transwell® inserts were fed every 2-3 days with 500 µl of co-culture medium in the basolateral compartment. For co-culture experiments, Wi38 fibroblasts were initially seeded followed by Calu-3 addition as previously described¹³². Transwell® inserts were initially inverted and seeded with 3.36×10^4 cells in 50 µl of Wi38 medium and incubated for 2 h to encourage cell attachment to the membrane. Thereafter, seeded inserts were returned to wells containing 1 ml of Wi38 medium and grown for 2 days. Two days after fibroblast seeding, inserts were seeded with Calu-3 cells on the apical side as previously described. For some experiments, Transwell® inserts seeded only with Wi38 fibroblast on the basolateral side were used and referred as fibroblast monoculture on Transwell® inserts.

2.2.3.4 Polarised Epithelial Culture on CHyA-B Scaffolds

For epithelial monocultures, Calu-3 epithelial cells were seeded on the apical side of 12 mm Snapwell® cell insert (VWR) as shown in Figure 2.1A, as previously described¹³². After chemical cross-linking, CHyA-B scaffolds of 15.6 mm-diameter and 1 mm in height were pinned into the frame of a Snapwell® cell insert and preconditioned using 3 ml of co-culture medium in the basolateral compartment and 400 µl in the apical compartment. The apical side of the scaffold was seeded with 5.65×10^5 Calu-3 cells in 100 µl of Calu-3 medium and incubated for three days. At day three of culture, medium from the apical compartment was removed to introduce ALI and samples were fed every 2-3 days with 2 ml of co-culture medium in the basolateral compartment. For co-culture experiments, the porous layer of each scaffold was pre-warmed with 3 ml of co-culture medium and seeded with 6×10^5 Wi38 cells in 50 µl of co-culture medium. Samples were incubated for 2 h to encourage cell attachment and supplemented with 3 ml of fresh medium thereafter. After two days of Wi38 cell

acclimatisation to the scaffold, scaffolds were then pinned into the frame of a Snapwell® cell insert and seeded with Calu-3 cells as previously outlined.

2.2.4 Evaluation of Epithelial Barrier Integrity

Transepithelial electrical resistance (TEER) values from epithelial monocultures and co-cultures on Transwell® inserts and CHyA-B scaffolds were quantified throughout the cell culture period to monitor epithelial barrier integrity. Fresh medium was added to the apical and basolateral compartments and TEER was measured using an EVOM voltohmmeter (World Precision Instruments, Hitchin, UK), STX-2 chopstick electrodes (World Precision Instruments) for Transwell® inserts and STX-3 chopstick electrodes (World Precision Instruments) for CHyA-B scaffolds. Transwell® inserts were filled with 500 µl of fresh co-culture medium in the apical compartment and 1 ml in the basolateral compartment, while 500 µl were added to the apical side of CHyA-B scaffolds and 2 ml to the basolateral compartment. Electrical resistance of cultures was measured on day 3, 5, 8, 10, 12 and 14 of culture. TEER values were calculated by subtracting the resistance of a cell-free scaffold or insert and corrected to the surface area (1.12 cm²) available for cell growth. TEER measurements for fibroblast monocultures on Transwell® inserts and CHyA-B scaffolds were performed as n = 2 due to the inability of fibroblast to form tight junctions and, therefore, produce relevant TEER values³³³.

2.2.5 In vitro Exposure

2.2.5.1 Reagent Selection and Cell Exposure Optimisation

Cell-seeded samples were exposed to a selection of drugs and toxicants in order to evaluate the toxicological and inflammatory responses in both CHyA-B and Transwell® cultures. LPS (derived from *Escherichia coli* and *Pseudomonas aeruginosa*(*P.aeruginosa*)) and bleomycin were selected to analyse responses to bacterial and drug toxicity, respectively. Bleomycin is an antibiotic used for the treatment of cancer with a significant side effect profile including fatal pulmonary toxicity and is a standard reagent used for preclinical respiratory toxicology³³⁴. Exposure conditions were optimised to provide an adequate toxicological and inflammatory challenge to epithelial cultures.

Non-polarised Calu-3 cells were exposed to *Escherichia coli* and *P.aeruginosa*-derived LPS at a range of concentrations (10, 1 and 0.1 µg/ml) for 24 h. LPS was

diluted to the relevant concentration in 500 µl of 1% v/v (serum starvation) and 10% v/v serum (non-serum starvation) Calu-3 medium and added to non-polarised Calu-3 cells. Interleukin-8 (IL-8) secretion was detected using ELISA MAX™ Deluxe Set Human IL-8 (BioLegend, London, UK) to evaluate the inflammatory response of Calu-3 cells following LPS exposure. Supernatants were taken and centrifuged at 1,200 rpm for 5 min and store at -20°C prior to use. A microwell plate was coated with 100 µl of 200X Coating Antibody diluted in 1X Coating Buffer A in deionised water overnight at 4°C. The plate was washed in between each step 4 times using wash buffer which contained Phosphate Buffer Solution (PBS) – 0.05% v/v Tween 20. Non-specific bindings were blocked with 200 µl 1X Assay Diluent A in PBS for 1 h at room temperature (RT). Samples were diluted in 1X Assay Diluent A and 100 µl of each sample was added and incubated for 2 h at RT. Following sample incubation, 100 µl of 200X Detection Antibody diluted in 1X Coating Buffer A was added and incubated for 1 h at RT. Afterwards, 100 µl of 1000X Avidin-HRP diluted in 1X Coating Buffer A was added to each well and incubated for 30 min at RT. 100 µl of Substrate C Solution was added, incubated for 10 min at RT protected from light and the reaction was then stopped using 1M Honeywell Fluka™ Sulfuric acid solution (Fisher Scientific). Absorbance was read at 450 nm and 570 nm. Cytokine release was calculated by subtracting the 570 nm value from the 450 nm and concentration was estimated using standard calibration curve following manufacturer's instructions. Once an optimal LPS dose and the exposure conditions were selected, polarised Calu-3 cells in Transwell® inserts under ALI conditions were exposed to 10 µg/ml of LPS derived from *P. aeruginosa* for 24 h and 48 h in the apical compartment using 1% serum Calu-3 medium and IL-8 release was evaluated.

Polarised Calu-3 cells on Transwell® inserts under ALI were exposed to bleomycin sulfate from *Streptomyces verticillus* at a range of concentrations (100, 10 and 1 µg/ml) for 24 h. Bleomycin was diluted to the appropriate concentration in 500 µl of Calu-3 medium. Cell metabolic activity, IL-8 secretion and TEER recovery were measured at day 15, 17, 19, 21 and 23 following exposure. IL-8 secretion was evaluated as previously reported. Cell metabolic activity was quantified using alamarBlue™ Cell Viability Reagent (Biosciences, Dublin, Ireland) as per the manufacturer instructions. Briefly, 1X alamarBlue™ was diluted in culture media, 1 ml was added on top of the cells and incubated for 2 h. Fluorescence was measured using 545 nm excitation and

590 nm emission wavelengths in a Tecan Infinite M Plex plate reader (Männedorf, Switzerland). TEER recovery values were defined using the following equation:

Equation 2. 1. Calculation of TEER recovery values.

$$\% \Delta TEER = \frac{\frac{TEER(f,t)}{TEER(i,t)}}{\frac{TEER(f,u)}{TEER(i,u)}} \times 100$$

where TEER (f,t) is the final TEER value measured of treated samples, TEER (i,t) is the mean TEER value measured before exposure on day 10, 12 and 14 of treated samples, TEER (f,u) is the final TEER value measured of untreated samples and TEER (i,u) is the mean TEER value measured before exposure on day 10, 12 and 14 of untreated samples.

2.2.5.2 Cell Toxicity Assays

Following the relevant apical exposure period to 10 µg/ml LPS and 100 µg/ml bleomycin in 1% serum Calu-3 medium to epithelial monocultures and co-cultures on Transwell® inserts and CHyA-B scaffolds, cell toxicity following exposure was assessed using alamarBlue™ Cell Viability Reagent and Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay (Fisher Scientific) to analyse the effect of the culture system on cell responses. Firstly, apical and basolateral samples were collected and lactate dehydrogenase (LDH) release was immediately assessed. Positive controls for each culture condition were used incubating epithelial cultures with 10 µl of Lysis Buffer. 50 µl of cell culture supernatant was incubated for 30 min at RT protected from light with 50 µl of Reaction Mixture, which was prepared by combining 600 µl of Assay Buffer Stock with Substrate Stock Solution. 50 µl of Stop Solution were added to each well and absorbance was read at 490 and 680 nm. To determine LDH activity, the 680 nm absorbance values were subtracted from the 490 nm values and LDH values were normalised to each untreated cell culture condition. Secondly, samples were incubated with alamarBlue™ to evaluate cell metabolic activity. 1X alamarBlue™ was diluted in culture media and 500 µl were added to the apical side of Transwell® inserts and CHyA-B scaffolds, while 500 µl and 2 ml of 1X alamarBlue™ were added to the basolateral compartments of Transwell® inserts and CHyA-B scaffolds respectively for 2 h. Fluorescence was measured using a 545 nm excitation and 590 nm emission

wavelengths and cell metabolic activity was normalised to each untreated cell culture condition.

2.2.5.3 Secretion of Pro-inflammatory Cytokines

Secretion of IL-8, interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF- α) following exposure to toxicants was assessed by enzyme-linked immunosorbent assay (ELISA). Apical and basolateral samples were collected and centrifuged at 1,200 rpm for 5 min before storage at -20°C prior to assay evaluation. Samples were assayed for IL-8, IL-6 and TNF- α using the ELISA MAX™ Deluxe Set Human IL-8, IL-6 and TNF- α (Biolegend) as previously outlined in Section 2.2.5.1.

2.2.6 Data Analysis

Quantitative data obtained were analysed using Microsoft Excel and GraphPad Prism 7.0 Software. For analysis between two groups, statistical difference was assessed by two-tailed Student t-test. For multiple groups, statistical difference between groups was assessed by 2-way Analysis of Variance (ANOVA). For analysis of the TEER measurements, repeated measures were performed as part of the 2-way ANOVA.

2.3 Results

2.3.1 Evaluation of Epithelial Barrier Integrity

Formation of a functional epithelial barrier was evaluated by measuring TEER from epithelial monocultures and co-cultures on Transwell® polycarbonate membrane inserts and CHyA-B scaffolds (Figure 2.2). Both epithelial monocultures and co-cultures were observed to produce TEER values higher than 600 Ωcm^2 , which characterises a functional epithelial barrier, with fibroblast monocultures showing negligible TEER < 50 Ωcm^2 (Figure 2.1B, Figure 2.2B). The inclusion of fibroblasts in Transwell® inserts significantly increased the average TEER from $750.2 \pm 75.13 \Omega\text{cm}^2$ in epithelial monocultures to $1029 \pm 126.9 \Omega\text{cm}^2$ in epithelial co-cultures. Fibroblast addition to CHyA-B scaffolds led to an increase in TEER values from $830 \pm 20.83 \Omega\text{cm}^2$ in epithelial monocultures to $912 \pm 75.94 \Omega\text{cm}^2$ in epithelial co-cultures but this was not statistically significant (Figure 2.1B, Figure 2.2B). A polarised and functional epithelial barrier was formed in both Transwell® inserts and CHyA-B scaffolds with similar values to those previously reported and the presence of fibroblasts in co-cultures appeared to improve epithelial barrier integrity¹³².

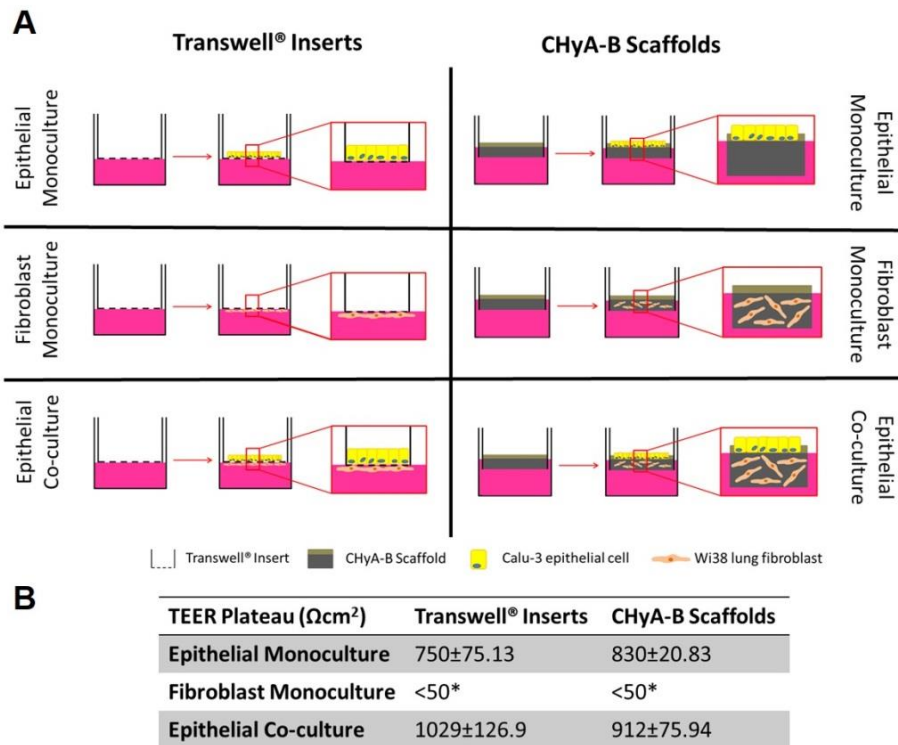


Figure 2. 1. Evaluation of epithelial barrier integrity in air liquid interface (ALI) culture models used for exposure studies. (A) Calu-3 epithelial monocultures, Wi38 fibroblast monocultures, and Calu-3 Wi38 epithelial co-cultures on Transwell® inserts and CHyA-B scaffolds grown under ALI conditions for 14 days. (B) Average TEER values of Calu-3 epithelial cell barriers following plateau of electrical resistance (day 10, 12, and 14). (* TEER measurements for fibroblast monocultures on Transwell® inserts and CHyA-B scaffolds were performed as $n = 2$). Results displayed as mean $\pm$ SEM ($n = 7$).

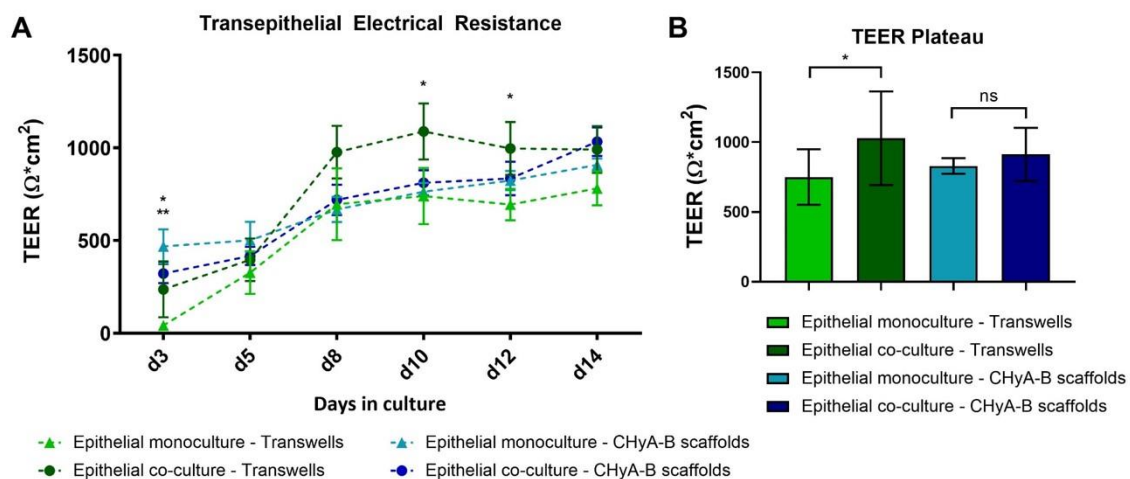


Figure 2. 2. Barrier integrity from epithelial monocultures and co-cultures on Transwell® inserts and CHyA-B scaffolds. (A) TEER values measured at day 3, 5, 8, 10, 12, and 14 from Calu-3 epithelial monocultures (light green) and Calu-3 Wi38 epithelial co-cultures (dark green) on Transwell® inserts; and Calu-3 epithelial monocultures (light blue) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds under ALI conditions. (B) Average TEER values of epithelial cultures following plateau of electrical resistance measured at day 10, 12, and 14. Results displayed as mean $\pm$ SEM ($n = 7$; * $p < 0.05$, ** $p < 0.01$).

2.3.2 Bacterial-mediated Toxicity and Inflammation

2.3.2.1 Optimisation of Bacterial Exposure Conditions

In order to optimise bacterial exposure conditions, non-polarised Calu-3 epithelial cells grown directly on culture dishes were exposed to LPS derived from *E. coli* and *P. aeruginosa* using three different concentrations: 10, 1, and 0.1 µg/ml for 24 h. Epithelial response to bacterial challenge was evaluated by assessing the release of IL-8, a key pro-inflammatory respiratory cytokine^{335, 336}. LPS derived from *E.coli* and *P. aeruginosa* led to a significant increase in IL-8 secretion in both apical and basolateral compartments when non-polarised epithelial Calu-3 cultures were exposed to 1 and 10 µg/ ml of LPS. However, *P. aeruginosa*-derived LPS was found to induce a more consistent dose response in comparison to *E. coli* and, therefore, was selected for further bacterial challenge optimisation (Figure 2.3A). Serum-starvation supported a more defined dose-response following LPS exposure in comparison to non-serum starvation conditions (Figure 2.3B). In order to determine optimal exposure time, polarised Calu-3 epithelial cells seeded on Transwell® inserts were challenged with 10 µg/ml of LPS (*P. Aeruginosa*) for 24 or 48 h from the apical compartment (Figure 2.3C). An exposure time of 24 h under serum starvation conditions led to a statistically significant increase in IL-8 secretion. Therefore, exposure of cells to 10 µg/ml LPS (*P. aeruginosa*) for 24 h under serum starvation conditions were used as standard exposure conditions for further bacterial challenge studies.

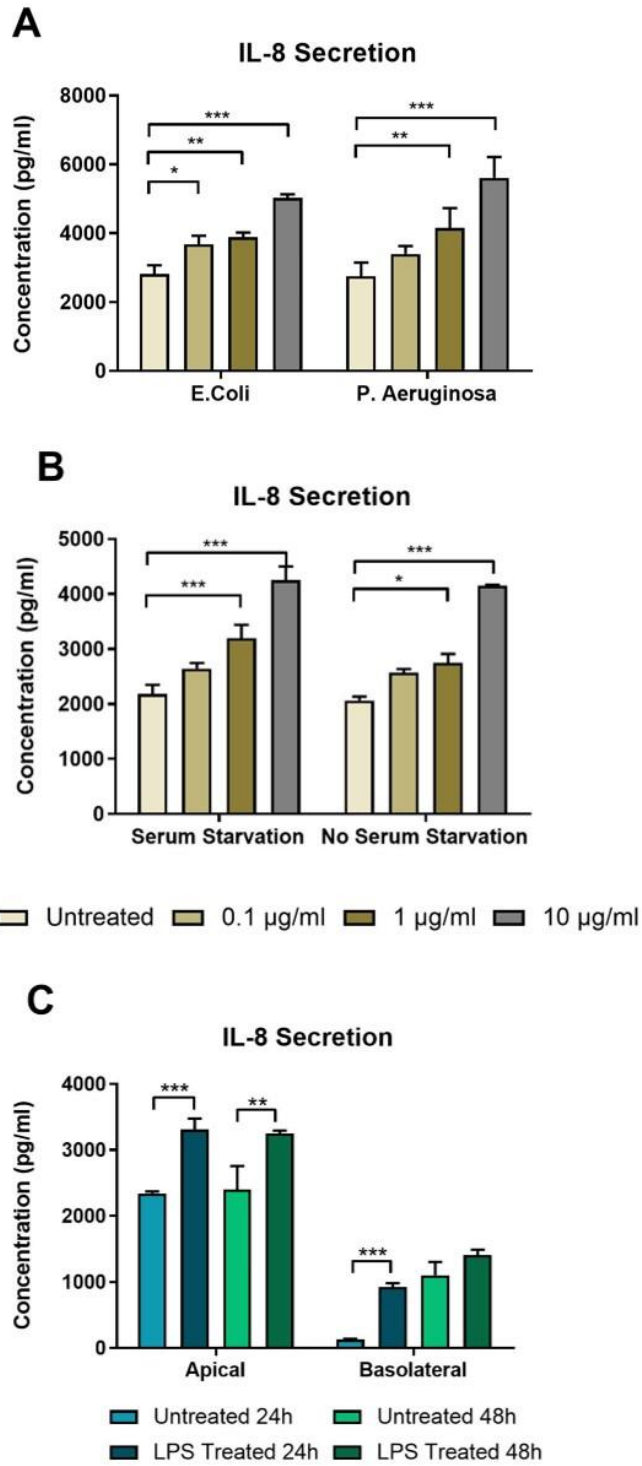


Figure 2. 3. Optimisation of LPS dose response in Calu-3 epithelial cells. (A) IL-8 secretion from non-polarised Calu-3 epithelial cells following LPS exposure with 10, 1, and 0.1 µg/ml of *E. coli* and *P. aeruginosa* derived LPS. (B) IL-8 secretion from non-polarised Calu-3 epithelial cells following LPS exposure 10, 1, and 0.1 µg/ml of *P. aeruginosa*-derived LPS in 1% (serum starvation) and 10% (no serum starvation) serum Calu-3 medium. (C) IL-8 secretion from polarised Calu-3 epithelial cells on Transwell® inserts following 10 µg/ml of *P. aeruginosa* derived LPS for an exposure time of 24 and 48 h. Cytokine release was quantified in both apical and basolateral compartments after bacterial challenge. Results displayed as mean ± SEM (n = 1; * p < 0.05, ** p < 0.01, *** p < 0.001).

2.3.2.2 Cell Toxicity After LPS Exposure in 2D and 3D

In order to compare responses of airway cells grown in 2D and 3D cultures the cell metabolic activity and LDH release of epithelial monocultures, fibroblast monocultures and epithelial co-cultures when grown on Transwell® inserts and CHyA-B scaffolds was evaluated 24 h after LPS exposure (Figure 2.4). Relative LDH release and cell metabolic activity from cultures grown on Transwell® inserts and CHyA-B scaffolds was assessed in the apical and basolateral compartments. As shown, cultures remained viable and metabolically active on both Transwell® inserts and CHyA-B scaffolds following LPS treatment relative to untreated cultures (Figure 2.4A,B). Equally, relative levels of LDH in treated cultures compared to untreated cultures grown on Transwell® inserts and CHyA-B scaffolds did not show any significant difference following LPS exposure (Figure 2.4C,D). TEER measurements following LPS exposure were performed to assess barrier integrity as uncontrolled cell death can cause TEER reduction³³⁷. Albeit an initial drop in barrier integrity compared to untreated cultures was detected following exposure, no significant differences were observed between epithelial cultures grown on Transwell® inserts and CHyA-B scaffolds with a sufficient recovery of barrier properties achieved after LPS exposure at day 23 (Figure 2.5).

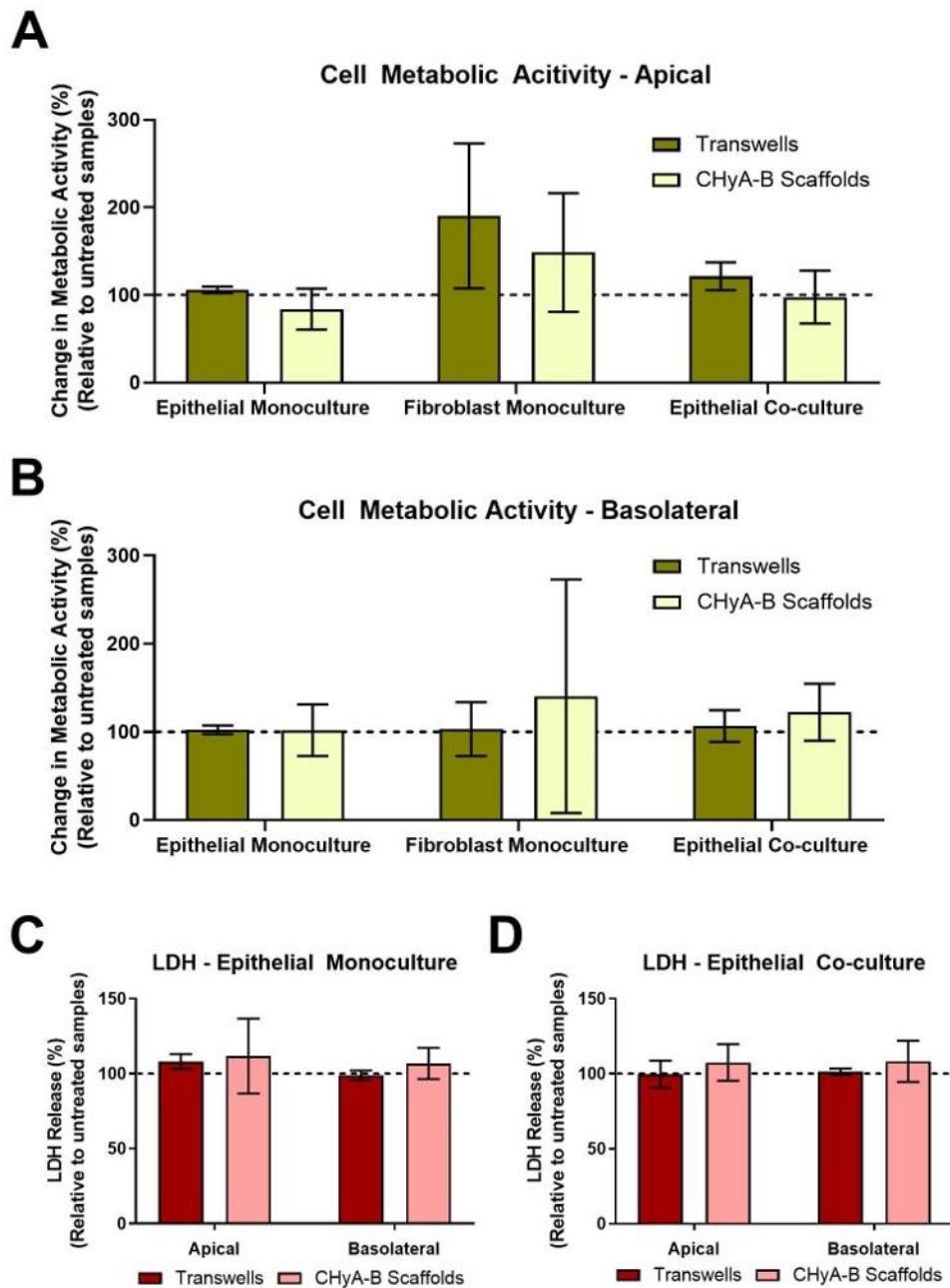


Figure 2. 4. Cell viability of epithelial monocultures and co-cultures grown on Transwell® inserts and CHyA-B scaffolds following bacterial challenge with 10 µg/ml of *P. aeruginosa*-derived LPS for 24 h. Apical (A) and basolateral (B) cell metabolic activity of Calu-3 epithelial monocultures, Wi38 fibroblast monocultures and Calu-3 Wi38 epithelial co-cultures on Transwell® inserts and CHyA-B scaffolds. Apical and basolateral LDH release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 epithelial co-cultures (D) on Transwell® inserts and CHyA-B scaffolds following LPS exposure. Changes in metabolic activity and LDH release are relative to untreated samples for each culture condition. Results displayed as mean ± SEM (n ≥ 3).

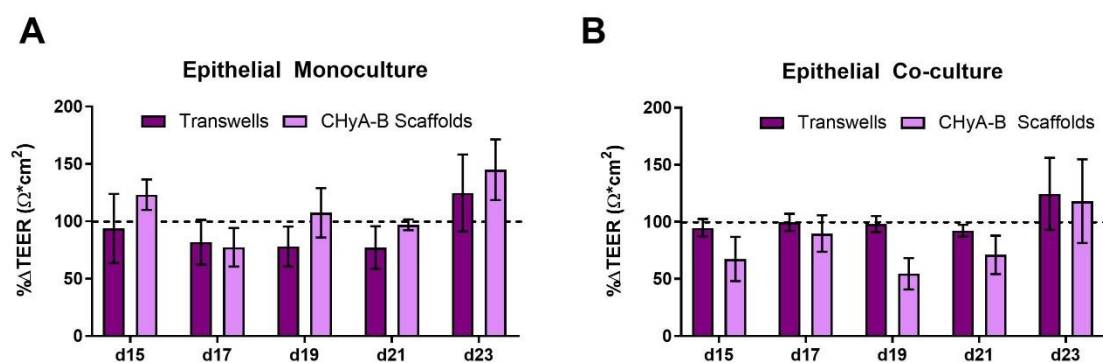


Figure 2. 5. TEER measurements following LPS exposure. TEER recovery of Calu-3 epithelial monocultures (A) and Calu3 Wi38 epithelial co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following bacterial challenge with 10 µg/ml of *P. aeruginosa*-derived LPS for 24 h. TEER recovery values normalised to the recovery of untreated cultures. Results displayed as mean ± SEM (n =3).

2.3.2.3 Inflammatory Response to LPS Exposure in 2D and 3D

To evaluate the inflammatory response to LPS in epithelial monocultures and co-cultures in Transwell® inserts and CHyA-B scaffolds, the release into the apical and basolateral compartments of the pro-inflammatory cytokines IL-8 and IL-6 was measured 24 h after exposure (Figure 2.6). All cultures grown on Transwell® inserts and CHyA-B showed an increase in IL-8 secretion following LPS challenge in comparison to the relevant untreated condition (Figure 2.6A,B). The apical secretion of IL-8 in epithelial monocultures was similar for those grown on Transwell® inserts and CHyA-B cultures. However, addition of co-culture conditions led to a greater increase in IL-8 secretion from CHyA-B compared to Transwell® cultures relative to untreated ones. Basolateral secretion of IL-8 from epithelial monocultures was significantly different between CHyA-B (2293 ± 934 pg in untreated cultures, 4191 ± 2555 pg in LPS-treated cultures) and Transwell® cultures (378 ± 349 pg in untreated cultures, 688 ± 335 pg in LPS-treated cultures) (Figure 2.6A). However, this differential basolateral profile in IL-8 secretion was not observed in Calu-3 Wi38 epithelial co-cultures (Figure 2.6B).

As for IL-8, release of IL-6 from Transwell® and CHyA-B epithelial monocultures was similar (Figure 2.6C). However, the addition of Wi38 fibroblasts led to increased IL-6 secretion from CHyA-B compared to Transwell® cultures (Figure 2.6D). Low basolateral levels of IL-6 were detected in untreated Transwell® and CHyA-B cultures. A significant increase in IL-6 secretion basolaterally from CHyA-B cultures following LPS exposure was detected which could not be observed in Transwell® cultures (Figure 2.6C). The addition of co-culture conditions led to significantly higher basolateral secretion of IL-6 release from CHyA-B cultures both untreated and LPS treated (6034 ± 4036 pg in untreated cultures, 8615 ± 4712 pg in LPS-treated cultures) in comparison to Transwell® cultures (37 ± 32 pg in untreated cultures, 81 ± 57 pg in LPS-treated cultures) (Figure 2.3D). TNF- α secretion from all cultures was not detectable.

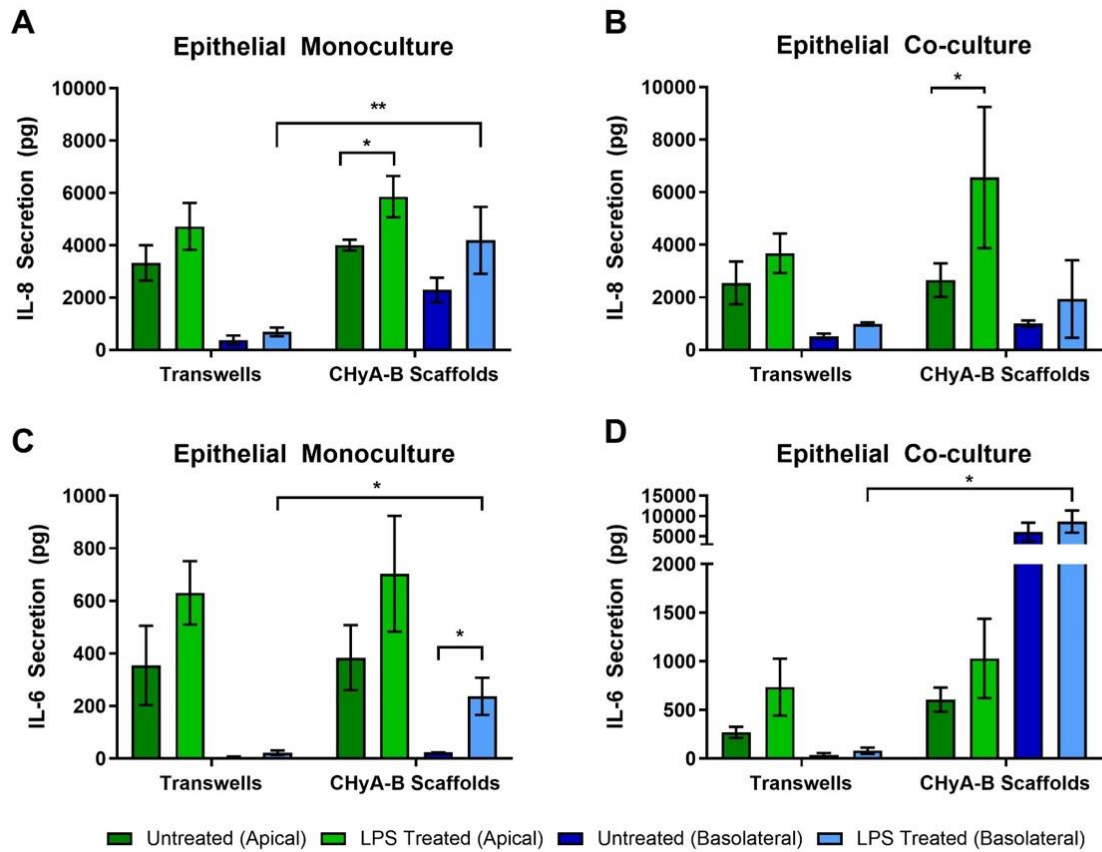


Figure 2. 6. Pro-inflammatory cytokine release profile from epithelial cultures following LPS exposure. Apical and basolateral IL-8 release from Calu-3 monocultures (A) and Calu-3 Wi38 co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following LPS exposure with 10 µg/ ml of *P. aeruginosa* derived LPS for 24 h. Apical and basolateral IL-6 release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 co-cultures (D) on Transwell® inserts and CHyA-B scaffolds following LPS exposure. Results displayed as mean ± SEM (n = 3; * p < 0.05, ** p < 0.01).

2.3.3 Drug-mediated Toxicity and Inflammation

2.3.3.1 Optimisation of Drug-mediated Exposure Conditions

As described in Section 2.2.5.1, bleomycin can be used as a model for drug-mediated respiratory toxicity in the creation of lung-injury animal models^{338, 339}. However, its use for *in vitro* testing is limited and has not been yet described using epithelial cells at the ALI interface³⁴⁰. Therefore, polarised Calu-3 epithelial cells grown on Transwell® inserts under ALI conditions were exposed apically for 24 h to 1, 10, and 100 µg/ml of bleomycin, an anticancer chemotherapy drug widely used in airway epithelial research to create induced lung injury models^{341, 342}. Cell metabolic activity, IL-8 secretion and TEER measurements from polarised epithelial cells were assessed at day 15, 17, 19, 21, and 23 following exposure to evaluate cell toxicity, airway inflammation and barrier integrity (Figure 2.7). Apical and basolateral cell metabolic activity from cultures treated with 10 and 100 µg/ml of bleomycin were significantly reduced relative to untreated cultures at all time points tested. The greatest decrease in cell metabolic activity was detected on day 23 following exposure to 100 µg/ml bleomycin with a $43.86 \pm 0.7\%$ apical and $37.89 \pm 2.1\%$ basolateral reduction compared to untreated cultures (Figure 2.7A,B). IL-8 secretion from polarised cultures was significantly increased relative to untreated samples at day 21 and 23 after 100 µg/ml bleomycin exposure (Figure 2.7C) and a significant decrease in TEER was observed at day 23 following 100 µg/ml bleomycin exposure (Figure 2.7D). Consequently, exposure to 100 µg/ml bleomycin for 24 h was selected as the standard exposure conditions for further drug-mediated toxicity challenge studies.

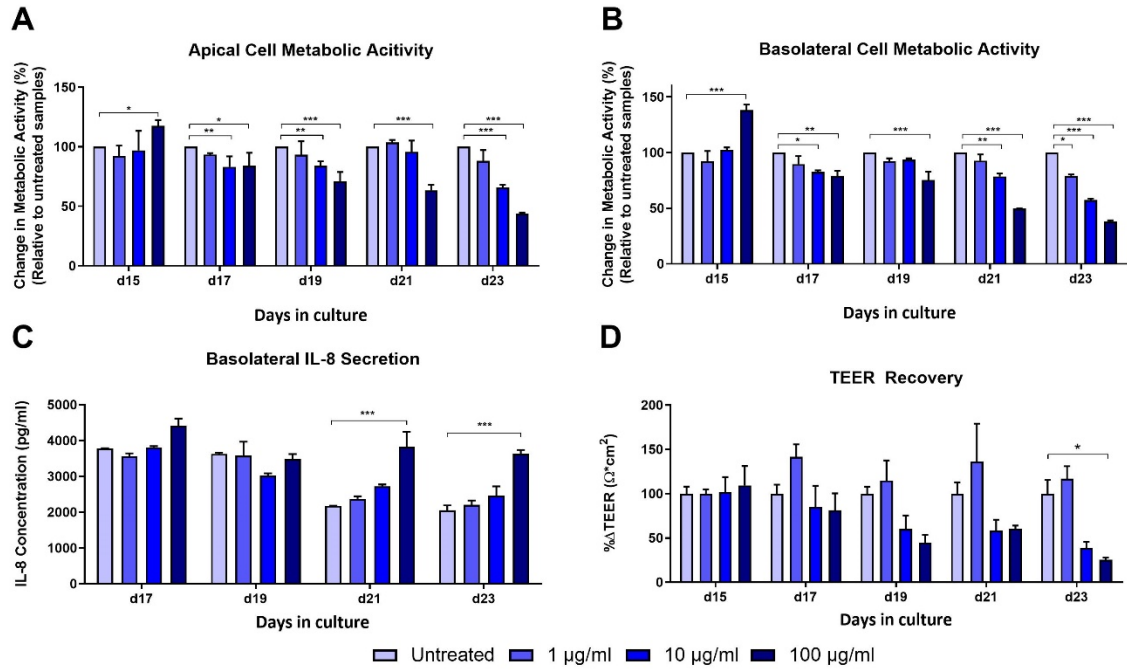


Figure 2. 7. Optimisation of drug-mediated challenge in polarised Calu-3 epithelial cells on Transwell® inserts with 1, 10, and 100 µg/ml of bleomycin for a 24 h exposure time. (A,B) Apical and basolateral cell metabolic activity from polarised Calu-3 epithelial cells. (C) Basolateral IL-8 secretion from polarised Calu-3 epithelial cells. (D) TEER recovery from polarised Calu-3 epithelial cells on Transwell® inserts TEER recovery values normalised to the recovery of untreated samples. Results displayed as mean ± SEM (n = 1; * p < 0.05, ** p < 0.01, *** p < 0.001).

2.3.3.2 Cell Toxicity after Bleomycin Exposure in 2D and 3D

Differential epithelial toxicological responses arising from exposure to bleomycin between culture conditions were compared by assessing the metabolic cell activity and LDH release of Transwell® and CHyA-B epithelial monocultures and co-cultures (Figure 2.8). Similar decreases in metabolic activity in the apical and basolateral compartment were seen in epithelial Transwell® and CHyA-B monocultures following drug-mediated challenge. In contrast, a significant drop in metabolic activity was observed in co-cultures grown on CHyA-B though not in Transwell® cultures (Figure 2.8A,B). In addition, significant differences were seen between basolateral LDH release from epithelial monocultures and co-cultures grown on CHyA-B scaffolds compared to Transwell® inserts post bleomycin exposure (Figure 2.8C,D). Transwell® epithelial monocultures showed increased LDH release ($168.47 \pm 70.4\%$) relative to untreated samples in comparison to a decrease ($84.44 \pm 4.86\%$) for CHyA-B epithelial monocultures (Figure 2.8C). This significant difference in cellular response to the bleomycin challenge between cultures was also observed for co-cultures grown on different supports (Figure 2.8D).

TEER changes following *in vitro* exposure can be used to detect cell damage and a decrease in barrier integrity³⁴³ using pre-exposure TEER values from Figure 2.2B and Equation 2.1. An initial reduction in TEER was seen in epithelial monocultures on CHyA-B scaffolds immediately after exposure. Furthermore, TEER measurements showed a slower recovery of epithelial barrier integrity for cells cultured on CHyA-B scaffolds in comparison to Transwell® inserts at day 21 and 23 after exposure relative to untreated cultures (Figure 2.9A). Interestingly, Transwell® and CHyA-B co-cultures were able to recover after bleomycin exposure showing no significant decrease in TEER in comparison to untreated co-cultures (Figure 2.9B).

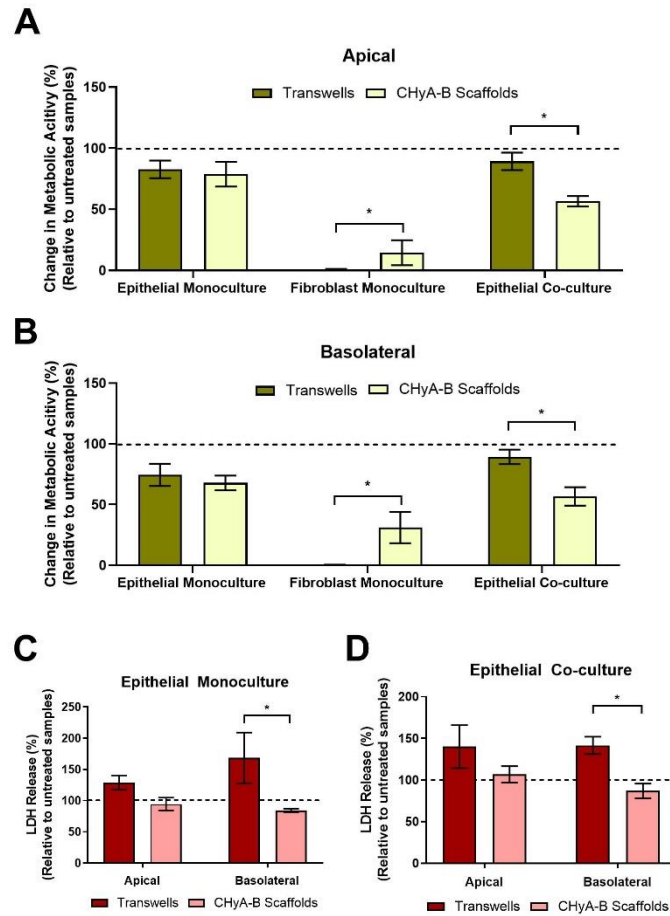


Figure 2. 8. Cell viability on Transwell® inserts and CHyA-B scaffolds following drug-mediated challenge. Apical (A) and basolateral (B) cell metabolic activity of Calu-3 epithelial monocultures, Wi38 fibroblast monocultures, and Calu-3 Wi38 epithelial co-cultures on Transwell® inserts and CHyA-B scaffolds following exposure to 100 µg/ml of bleomycin for 24 h and measured at day 23 in culture. Apical and basolateral LDH release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 epithelial co-cultures (D) on Transwell® Inserts and CHyA-B scaffolds following bleomycin exposure. Changes in metabolic activity and LDH release are relative to untreated samples for each culture condition. Results displayed as mean ± SEM (n ≥ 3; * p < 0.05).

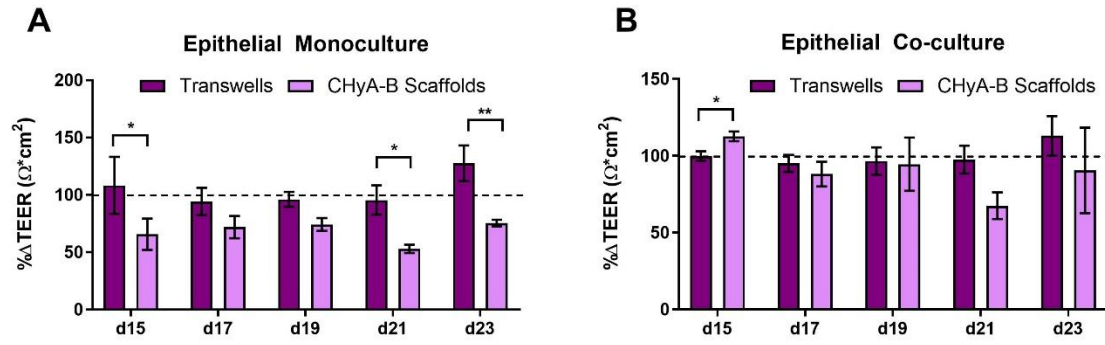


Figure 2. 9. TEER measurements following drug-mediated challenge. TEER recovery of Calu-3 epithelial monocultures (A) and Calu3 Wi38 epithelial co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following challenge with 100 µg/ml of bleomycin for 24 h measured at day 15, 17, 19, 21, and 23 in culture. TEER recovery values normalised to the recovery of untreated samples for each culture condition. Results displayed as mean ± SEM (n = 3; * p < 0.05, ** p < 0.01).

2.3.3.3 Inflammatory Response to Bleomycin Exposure in 2D and 3D

To further assess the effect of bleomycin on epithelial cultures, the production of pro-inflammatory cytokines in response to drug-mediated challenge was measured at day 23 after exposure (Figure 2.10). IL-8 secretion was found to increase following bleomycin treatment in all Transwell® and CHyA-B cultures (Figure 2.10A,B). However, basolateral levels of IL-8 were increased significantly from CHyA-B epithelial monocultures in comparison to Transwell® monocultures in both untreated and bleomycin-treated cultures (Figure 2.10A). This relative increase in IL-8 expression when cells were cultured on the CHyA-B scaffolds was also seen for epithelial co-cultures (Figure 2.10B). Most notably, the only statistically significant increase in IL-8 expression post-bleomycin treatment was detected in cultures grown on CHyA-B scaffolds (Figure 2.10A,B).

Bleomycin exposure increased the secretion of IL-6 in comparison to the relative untreated controls, although this increase was only significant for CHyA-B monocultures (Figure 2.10C,D). There were no significant differences in apical secretion of IL-6 between Transwell® and CHyA-B epithelial monocultures, but inclusion of Wi38 fibroblasts led to a two-fold increase of IL-6 secretion in Transwell® and CHyA-B co-cultures (Figure 2.10C,D). Basolateral secretion of IL-6 from Transwell® epithelial monocultures remained low with significantly higher secretion observed in CHyA-B scaffolds (Figure 2.10C). Furthermore, basolateral IL-6 levels from CHyA-B epithelial co-cultures were significantly higher in comparison to Transwell® epithelial co-cultures, but no significant increase in IL-6 was seen following bleomycin exposure (Figure 2.10C). TNF- α secretion from all cultures was not detectable.

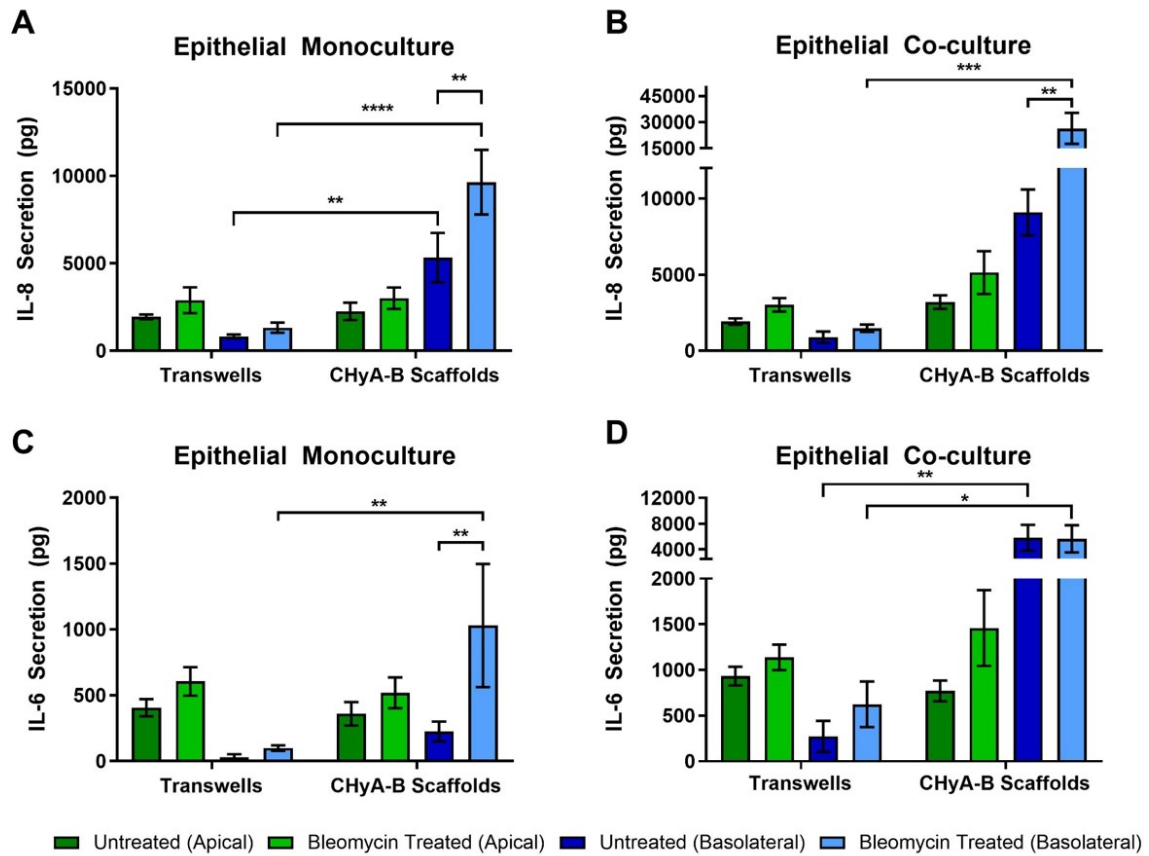


Figure 2. 10. Pro-inflammatory cytokine release profile from epithelial cultures following drug-mediated challenge. Apical and basolateral IL-8 release from Calu-3 epithelial monocultures (A) and Calu-3 Wi38 co-cultures (B) on Transwell® inserts and CHyA-B scaffolds following challenge with 100 µg/ml of bleomycin for 24 h measured at day 23 in culture. Apical and basolateral IL-6 release from Calu-3 epithelial monocultures (C) and Calu-3 Wi38 co-cultures (D) on Transwell® inserts and CHyA-B scaffolds following bleomycin exposure. Results displayed as mean ± SEM (n = 3; * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

2.4 Discussion

The use of polarised epithelial models of the tracheobronchial region, primarily grown on filter inserts under ALI conditions, are well-established and are used to mimic *in vivo* physiological processes and inhalation studies dynamics⁴. However, improvements in these epithelial models are needed to fully reflect epithelial responses to challenges and treatments *in vivo* in order to facilitate drug development and to better understand cellular responses in the airway. The use of conventional 2D models, such as cell culture inserts, e.g., Transwell®, are limited by the lack of an ECM-like 3D environment that better recapitulates epithelial responses to biochemical and biophysical stimuli³⁴⁴. Moreover, conventional airway *in vitro* models often do not fully recapitulate the multicellular *in vivo* environment in the lungs as single cell type cultures are commonly used. As with other human tissues, the lining of the lower respiratory tract contains a wide variety of cell types that can be better recapitulated using co-culture models of the airway. In this way, complex cell-to-cell communication and crosstalk between cell types can better represent epithelial responses against toxicological and inflammatory process and match more closely those of a complex *in vivo* system^{325, 345, 346}. Therefore, the purpose of this Chapter was to evaluate a previously developed TE bilayered scaffold of the tracheobronchial region as an *in vitro* model to more closely mimic the epithelial responses against bacterial and drug-mediated toxicological and inflammatory challenges¹³².

Polarised Calu-3 epithelial cells grown on Transwell® inserts have become a standard model to study airway cell interactions to inhaled pathogens as well as other stimuli causing inflammation and epithelial cell toxicity⁸⁷. LPS has been widely used as a positive control to generate inflamed models of the tracheobronchial region, but standard exposure conditions have not been established in the literature. This hampers the development of robust inflammatory *in vitro* models of the respiratory tract and accurate comparison between them³⁴⁷. Herein, a systematic optimisation of challenge conditions was undertaken to study the effect of LPS on the secretion of the pro-inflammatory cytokine IL-8 using different conditions including different concentrations, serum concentrations in media and exposure times in non-polarised and polarised Calu-3 epithelial cells on Transwell® inserts (Figure 2.3). Our findings showed that a concentration of 10 µg/ml of *P. aeruginosa*-derived LPS in serum starvation conditions for 24 h was suitable for the creation of a robust bacterial

challenge in a polarised epithelial airway model. *P. aeruginosa*-derived LPS was chosen over *E. Coli* due to a better dose-response observed, although both LPS led to a significant increase in IL-8 secretion compared to untreated cultures. Serum starvation conditions as a more defined dose-response was supported and to avoid serum interferences during cytokine analysis.

Drug-mediated toxicity and inflammation of the airway epithelium are typically studied using bleomycin lung injury animal models. Bleomycin is a potent anticancer drug that causes pulmonary fibrosis due to changes in fibroblast phenotypes³⁴⁸. These *in vivo* models are associated with ethical concerns and sometimes poor correlation with human responses due to anatomical and physiological differences between humans and animal models³⁴⁹. Therefore, new *in vitro* models for drug-mediated injury on human tissues could provide much needed tools for translation. To the best of this author's knowledge, this is the first report of the effect of bleomycin exposure on polarised Calu-3 epithelial cells. Again, the conditions of exposure were systematically optimised. Decreased metabolic activity, increased IL-8 secretion and poor TEER recovery following 100 µg/ml bleomycin exposure on Calu-3 cells grown on Transwell® inserts was determined to provide suitable challenge conditions (Figure 2.7). These cellular responses following exposure *in vitro* are similar to those seen in bleomycin-induced lung injury models, in which inflammation and increased cell apoptosis are seen in the first 7 days after intratracheal administration³³⁹. Inflammation observed in this 2D polarised model also resembles the increased secretion of cytokine from murine models with high release of pro-inflammatory cytokines 9 days after exposure³³⁸.

ALI culture conditions for epithelial cells have been shown to support more realistic cell-to-cell interactions and cell responses towards potential lung toxicants compared to non-polarised models, which facilitates better assessment of therapeutics and formulations^{20, 350, 351}. For example, polarised models allow for maintenance of a mucus layer over the epithelial monolayer, a key feature involved in the deposition and uptake of inhaled nanoparticles³⁵². Overall, these ALI polarised models better predict cell toxicity, inflammation and barrier integrity, the main outputs herein examined after epithelial exposure^{346, 353}. It was hypothesised that these polarised models could be further improved by culturing epithelial cells in a 3D environment that resembles the ECM present in physiological conditions, allowing for cell-ECM interaction that is not

effectively modelled in 2D models³⁵⁴. The presence of an ECM-like environment better recapitulates tissue architecture as well as providing mechanical and biomechanical cues. Moreover, it has been widely reported that cell to ECM interactions establish a 3D communication network which allows for tissue homeostasis maintenance modulating cell proliferation, migration, and apoptosis^{233, 234}. Therefore, it is crucial to include 3D environments in *in vitro* airway models. The use of co-cultures or even tri-cultures, e.g., lung fibroblasts, bronchial fibroblast, macrophages and mesenchymal stem cells in airway *in vitro* models has been explored over the last decade to better understand crosstalk between epithelial cells and under-lying cell populations^{205, 212, 228, 243, 346, 355}. For instance, inclusion of fibroblasts have been reported to increase proliferation and differentiation of co-cultured epithelial cells, while inclusion of a variety of immune cells allows for better interpretation of crosstalk at the epithelial barrier^{106, 242, 356}. Inclusion of multiple cell types in an ECM-like environment to establish 3D epithelial models could potentially help understand and represent more realistic epithelial responses following external stimuli.

The work in this Chapter described the culture of airway epithelial cells on a TE model that mimics the tracheobronchial region and surrounding tissues¹³². Similar epithelial barrier formation for Transwell® and CHyA-B epithelial monocultures was detected, which correlates with previous findings for this scaffold¹³². However, inclusion of co-cultures conditions in Transwell® cultures led to increased TEER values compared to monocultures, which was not seen in CHyA-B cultures as barrier formation remained similar to monoculture of epithelial cells (Figure 2.2). Reported TEER values from epithelial cultures in CHyA-B matched those reported previously for human primary epithelial cells and values observed for Calu-3 cells in conventional 2D Transwell® insert models^{132, 230, 357, 358}. Reduction of TEER values upon culture on 3D matrices has also been described in airway and intestinal mucosal models and proved to be a more accurate reflection of *in vivo* barrier formation while inclusion of supporting cell types leads to a faster barrier formation and increased TEER readings^{106, 359, 360}. It has been described that a reduction of TEER upon epithelial culture in 3D environments arises from the formation of less strong tight junctions, but this does not affect barrier formation or represents weaker epithelial barrier integrity overall³⁶¹. Barrier integrity (TEER values) did not fully recover in CHyA-B epithelial monocultures after exposure to bleomycin (Figure 2.9A). Bleomycin has been shown to impair barrier function and

cause severe lung damage and toxicity in animal models as well as in mice-derived lung epithelial cells³⁶²⁻³⁶⁵. Albeit dosing comparison between *in vivo* and *in vitro* models can be challenging to interpret, the use of an extended concentration range of 0–1000 mU/ml bleomycin showed comparable decreased viability in an *in vitro* model comprising lung epithelial cells as herein reported, which would result in reduced TEER values due to damaged barrier properties³⁶⁵. Interestingly, inclusion of Wi38 fibroblasts in CHyA-B cultures led to greater TEER recovery relative to untreated samples highlighting the importance of co-culture models to better mimic epithelial responses and epithelial-fibroblasts crosstalk in 3D airway models³⁴⁵. The presence of an adjacent fibroblast sublayer has been described to influence barrier formation via growth factor secretion and deposition of ECM components in a human intestinal mucosa model³⁶⁶. Therefore, it is expected that epithelial cells cultured in the presence of lung-derived fibroblast will develop enhanced barrier properties towards toxic stimuli as the protective role of fibroblast in airway cells has already been described³⁶⁷.

Differential toxicological responses were detected between Transwell® and CHyA-B cultures after bleomycin exposure with higher toxicity seen in CHyA-B epithelial co-cultures, demonstrating a greater sensitivity to this challenge. However, the release of LDH from epithelial monocultures and co-cultures grown on CHyA-B scaffolds was maintained relative to untreated cultures. In contrast, a spike in LDH release was observed in Transwell® monocultures and co-cultures post-bleomycin treatment. LDH is released from cells following tissue damage, therefore it was hypothesised that Transwell® cultures were damaged following bleomycin treatment. According to metabolic activity and TEER measurements, the epithelial integrity was maintained and therefore LDH levels were higher relative to untreated samples. However, the low LDH release observed from CHyA-B cultures after bleomycin exposure might be explained by the greater disruption and cell damage elicited by the drug in these cultures. It was speculated that fewer cells were present in scaffolds compared to Transwell® cultures after exposure as evidenced by lower metabolic activity and the inability to recover TEER values in epithelial monocultures from CHyA-B scaffolds (Figure 2.8, Figure 2.9). This behaviour of increased cell sensitivity to toxicants in 3D versus 2D models has already been reported and it is hypothesised that cells grown in 3D environments could offer more sensitive responses when investigating nanoparticle effects for example on epithelial cells^{31, 368}. Although it is challenging to

draw similarities with actual human data due to its scarcity, it is believed that the higher sensitivity of 3D models might arise due to reduced nutrient supply compared to 2D as well as higher basal oxidative stress, which might better represent *in vivo* conditions^{31, 369}.

Development of 3D models of the airway also has the potential to better represent immune competent models of the airway by combining airway cells with lung and bronchial fibroblasts³⁷⁰⁻³⁷². Secretion of cytokines from epithelial cells grown on 3D supports and under ALI conditions is linked to the formation of a fully functional epithelium and better represents fibroblast-epithelial interactions²²⁸. Apical cytokine secretion from 2D cultures was higher in comparison to basolateral secretion as previously reported³⁷³, but this was not seen in 3D cultures. Moreover, increased basolateral secretion of pro-inflammatory cytokines was detected in CHyA-B cultures, in comparison to Transwell® cultures following exposure to LPS and bleomycin. In this manner, basolateral expression was able to better detect changes in cytokine secretion, highlighting the need to further investigate the role of polarised release of pro-inflammatory mediators when assessing inflammatory responses to inhaled toxicants/formulations. Increased cytokine secretion upon culture of epithelial cells in 3D has been demonstrated to better resemble *in vivo* situations and recruitment of immune cells after epithelial damaged is driven by secretion of cytokines and other mediators³⁷⁴⁻³⁷⁸. Increased detection of cytokine secretion in 3D models is hypothesised to arise from better representation of signalling mechanisms that can remain undetected or underrepresented in 2D models^{344, 379, 380}. Therefore, increased cytokine secretion from CHyA-B scaffolds could better mimic airway cells crosstalk and immune response to external stimuli which can be explained due to the role of ECM environment regulating cytokine secretion³⁸¹. Inclusion of lung derived fibroblasts led to decreased basolateral levels of IL-8 following LPS stimulation compared to monocultures on CHyA-B scaffolds (Figure 2.10A,B). This reduced airway inflammatory response upon fibroblast incorporation in a 3D environment matches the protective role of fibroblast in the airways³⁸². On the other hand, co-culture conditions lead to higher pro-inflammatory cytokine secretions after bleomycin exposure (Figure 2.10B,D). Here we outline a model that could provide better understanding of airway pathology and responses to external stimuli, which could be easily detected by monitoring the secretion of key lung pro-inflammatory cytokines.

Furthermore, inclusion of other relevant cell types modulating the immune response after toxicant inhalation should be investigated to better understand cellular responses. Establishing multicellular models on scaffolds resembling ECM and the interaction of cells with their surrounding environment could hold promise to better analyse the inflammation profile following irritant exposure. The development of predictive *in vitro* models that are able to detect early signs of toxicity and adverse inflammatory cues would allow for improved development of inhaled therapeutics without failure during preclinical assessment due to unidentified side effects³⁸³.

2.5 Conclusion

The goal of this Chapter was to evaluate the feasibility of CHyA-B scaffolds as an improved *in vitro* model to study toxicological and inflammatory responses from epithelial cultures upon exposure to irritant substances. We established standardised challenge conditions for bacterial and drug-mediated toxicity and inflammation, which will be important in comparing relevant *in vitro* airway models as they emerge. The toxicological profile and secretion of key inflammatory mediators after exposure to stimuli was shown to be significantly different when a mature tracheobronchial epithelium was grown in collagen-based scaffolds (3D) compared to standard filter cultures (2D). These results highlight the importance of modelling the ECM-like environment present in airway tissues as well as the critical role for co-cultures to properly interrogate responses in *in vitro* models of the airways.

The use of TE 3D models of the airway has the potential to increase our understanding of epithelial responses to toxicological and immunological stimuli and therefore act as a screening tool for drug development by providing reliable tools to study inhaled therapies toxicity and inflammatory profile in an early stage of clinical development.

Chapter 3

**Assessment of Nanoparticles using a Co-culture
Tissue-Engineered *In Vitro* Model of the Airway**

3.1 Introduction

Inhalation is the preferred route of drug delivery for a range of therapeutics targeting respiratory diseases. It facilitates direct delivery to the diseased organ, minimises systemic drug exposure, leads to less severe adverse effects and supports a faster onset of action when compared to other routes of administration including oral and parenteral administration^{324, 384}. Despite many drug candidates demonstrating excellent safety and efficacy during pre-clinical evaluation, the majority suffer from poor clinical success due to minimal therapeutic effectiveness^{15, 385}. Failure to further progress through the clinical assessment process can be traced to the poor understanding of biological barriers, and in the case of inhaled treatments for airway diseases, the respiratory epithelium³⁸⁶. Not only do many novel therapies fail to progress due to lack of efficacy, but also due to unacceptable toxicity and/or inflammatory effects that can remain undetected during pre-clinical assessment, in spite of the time-consuming and expensive toxicity testing commonly employed³¹.

To date, the use of animal models remains the gold standard to assess the efficacy and toxicity of novel inhaled drugs. Although animal testing is currently the only alternative to provide a full systematic overview of a drug's effects on a model organism, the use of *in vitro* testing has seen rapid development during the last decades, supported by the principle of the three Rs – reduction, refinement and replacement^{387, 388}. Moreover, species-specific differences can also play a significant role in the poor extrapolation from animals to humans³⁸⁶. Although safety and efficacy in animal models is carried out during the preclinical assessment process and there are continuous efforts to reduce animal testing, it is of crucial importance to account for animal testing drawbacks that might hinder novel therapies development³⁸⁶. For example, rodent models do not fully represent the effect of the oropharynx in reducing the effective therapeutic dose reaching human lungs and large animal models present ethical and financial drawbacks as well as extensive difficulties in bridging animal data to human applications due to metabolic, physiological and anatomical differences^{13, 389}. Several examples can be found across literature for the optimisation of the 3R principle. New *in vitro* models of spinal cord injury correlate with *in vivo* findings and can be actively applied for the screening of new purposed therapeutics³⁹⁰. For example, models using induced pluripotent stem cells (iPSC) have been demonstrated to model chemotherapy-induced cardiotoxicity and can be successfully employed to

determine the patient's most optimal treatment without the need for further animal testing³⁹¹.

Therefore, there is an important need to develop more accurate and predictive models of the human airway that will boost the translation of novel nanomedicines and will not fail during *in vivo* assessment in the regulatory process. *In vitro* models offer an alternative for animal exposure testing and can be tailored to provide high-throughput screening of novel therapies³⁸⁶. Models of the tracheobronchial region are commonly employed for pre-clinical evaluation as it is estimated that around 25% of therapeutically inhaled particles deposit in this section of the respiratory tract³¹. Several different bronchial cell types are used in these models including Calu-3 and 16HBE14o cells to fully represent tight junction formation in the airway epithelium²⁴². A549 and BEAS-2B are also commonly used airway cell lines but their use has been limited due to the lack of tight junction formation, mucus secretion and therefore, poor drug absorption modelling⁸³. Moreover, the alveolar characteristics of A549 cells makes them more suitable for models of the alveolar region of the lungs. The use of single cell type *in vitro* models grown on flat two dimensional (2D) cell culture dish surfaces does not allow for epithelial polarisation and certainly does not mimic the complex cell-cell and cell-extracellular matrix (ECM) interactions of the human lungs³⁹².

Tissue-engineered (TE) models of the airway aim to address some of these drawbacks by growing multiple airway cells in a three dimensional (3D) environment to provide physiologically relevant cellular responses. 3D models have proven to be superior to 2D *in vitro* models by better recapitulating the 3D environment. As an example on how 3D outperforms 2D models, they have been shown to modulate cell proliferation, resulting in reduced activity of chemotactic drugs and to reduce cytotoxicity to nanoparticles when compared to 2D models^{393, 394}. Introduction of co-culture conditions in respiratory models, on the other hand, has been demonstrated to alter particle cellular uptake and cell sensitivity in comparison to monoculture models³⁹⁵. Co-cultures have also been shown to modulate nanoparticle induced cellular toxicity while increasing the secretion of inflammatory markers following exposure to silica nanoparticles^{31, 396}.

The application of improved *in vitro* models of the airway for the assessment of drug candidates not only has the potential to reduce the requirement for animal testing but also increases the rate of translation of novel therapies that fail during pre-clinical and clinical evaluation³⁹⁷. The incorporation of accurate and high throughput *in vitro* testing holds the potential to screen for a large variety of therapeutics and elucidate therapy effectiveness early in the development process while enabling early toxicity detection¹³. 3D models of the airway hold promise for advanced, fast and cost-effective drug screening for toxicity in physiologically relevant conditions which will ultimately result in better clinical translation of novel inhaled nanotherapies^{225, 398}.

In recent years, several nanoparticles have been proposed as drug-delivery systems in biomedical applications. For instance, transferrin-modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles have been proposed as new alternatives in chemotherapy³⁹⁹. Wrapping of PLGA nanoparticles with bovine serum albumin has shown promising anticancer activity using *in vitro* and *in vivo* models without eliciting toxicity⁴⁰⁰. Carbon nanomaterials such as carbon nanotubes (CNTs) are highly variable in geometries, which can be specifically tailored and have been recently applied to obtain more efficient drug delivery or as potential treatments for cancer when their toxicity is drastically reduced and allow for biomedical applications⁴⁰¹. Apart from well-known and characterised nanomaterials, wide progress has been made in the development of novel nanomaterials that can allow for enhanced drug delivery and better therapeutic efficacy. For example, recently developed bioinspired star-shaped nanoparticles –G5 nanoparticles- have been successfully applied as gene delivery platforms for mesenchymal stem cells (MSCs), bone regeneration and for the delivery of angiogenic factors⁴⁰²⁻⁴⁰⁴. Despite the enormous range and variety of biomaterials that have been bioengineered for the development of novel nanoparticles for drug delivery applications, the translation of nanomedicines available to patients is well below official projections⁴⁰⁵. The crucial differences between the animal models used for the development of these novel nanomedicines and human physiology as well as patient variability leads to low translation of nanomedicines⁴⁰⁶. Therefore, development of more advanced *in vitro* models could significantly boost the translation of new nanomedicines by providing testing platforms for safety and toxicity while allowing tailoring of patient-specific treatments for individualised medicine⁴⁰⁵.

3.1.1 Aims and Objectives

In Chapter 2, collagen and hyaluronic acid (CHyA-B) scaffolds were validated as an improved *in vitro* model of the tracheobronchial region that provides physiologically-relevant cellular responses against toxic and inflammatory stimuli. The aim of this Chapter was to use the previously developed TE co-culture model as an advanced platform to assess the safety and toxicity of nanomaterials (PLGA and CNTs) as well as novel inhaled nanomedicines (G5 miR and NC nanoparticles). The term nanoparticle will be used to refer to both nanomaterials and nanomedicines under study in this Chapter. For these purposes, epithelial responses from cells grown in co-culture conditions in CHyA-B scaffolds were compared to the current standard model of epithelial monocultures on filter inserts. LPS and bleomycin-induced epithelial responses optimised in Chapter 2 were used as positive controls for epithelial toxicity and inflammation.

The specific objectives were:

- To determine the short-term differences in epithelial toxicological and inflammatory responses after exposure to the nanoparticles between 2D and CHyA-B scaffold cultures.
- To determine long-term differences in epithelial toxicological and inflammatory responses after exposure to nanoparticles between 2D and CHyA-B scaffold cultures.
- To examine changes in epithelial barrier integrity across epithelial cultures following nanoparticle exposure cultured on CHyA-B scaffolds.

3.2 Materials and Methods

3.2.1 Materials

Unless specified in the text, all materials and reagents were supplied by Sigma-Aldrich (Arklow, Ireland).

3.2.2 Scaffold Fabrication

3.2.2.1 Collagen and Hyaluronic Acid (CHyA) Film Fabrication

Collagen and hyaluronic acid (CHyA) films were fabricated using a modification of a previously described method as outlined in Section 2.2.2^{132, 328}. A suspension of 0.5% w/v type I bovine tendon collagen (Collagen Solution, Glasgow, UK) and 0.004% w/v hyaluronic acid sodium salt from *Streptococcus equi* in 0.5 M acetic acid (AcOH) (Fisher Scientific, Ballycoolen Ireland) was blended at 4°C and 15,000 rpm for 3.5 h using an Ultra Turrax T18 Overhead blender (IKA Works Inc., Wilmington, NC). The CHyA slurry was degassed under a vacuum to remove air bubbles created during the homogenisation process, it was pipetted onto a 6x6 cm² polytetrafluoroethylene plate and left 48 h under an air flow in a fume hood to produce a thin and transparent CHyA copolymer film 20 µm in thickness.

3.2.2.2 Collagen and Hyaluronic Acid-bilayered (CHyA-B) Scaffold Fabrication

CHyA-B scaffolds were manufactured by freeze-drying CHyA films combined with CHyA slurry using a customised metal mould as previously described^{132, 329}. CHyA films were rehydrated for 2 h in 0.5 M AcOH, cut into 6 cm x 6 cm strips and placed onto the base of a stainless steel grade 304 SS pan. 4 ml of CHyA slurry was pipetted on top of the film and the slurry-film combination was freeze-dried using a customised anneal cycle that initially froze the combination to -20°C before being heated to -10°C and held for 24 h prior to sublimation³³⁰. After freeze-drying, CHyA-B scaffolds were cross-linked using a dehydrothermal (DHT) process at 105°C during 24h at 50 mTorr (VacuCell 22, MMM, Planegg, Germany)³³¹.

3.2.2.3 Chemical Cross-linking

CHyA-B scaffolds were cut into discs with a diameter of 15.6 mm², hydrated in Dulbecco's Phosphate Buffer Solution (DPBS) for 30 min and chemically cross-linked

using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC). Briefly, each scaffold was cross-linked for 2 h under UV light using 2 ml of EDAC solution which consisted of 6 g mmol EDAC per gram of scaffold and N-Hydroxysuccinimide (NHS) in a 5:2 molar ratio of EDAC:NHS^{330, 332}. Scaffolds were washed with DPBS to remove any residual toxic product and stored in DPBS at 4°C prior to use.

3.2.3 Cell Culture

3.2.3.1 Cell Selection and Culture Media

The Calu-3 bronchial epithelium cell line (ATCC, Middlesex, UK) was cultured in 175 cm² CELLSTAR® cell culture flasks following revival from liquid nitrogen. Calu-3 cells were seeded at a density of 1×10^6 cells per T175 cell culture flask and were passaged when they reached confluency using 0.25% Trypsin-EDTA solution. Calu-3 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% v/v foetal bovine serum (FBS) (Biosera, Ringmer, UK), 2 mM L-glutamine, 14 mM sodium bicarbonate and 100 U/ml penicillin/streptomycin; this was referred to as Calu-3 medium. Cells were used between passages 20-38. The Wi38 human embryonic lung fibroblast cell line (ATCC) was cultured in 175 cm² CELLSTAR® cell culture flasks following revival. Wi38 cells were seeded at a density of 1×10^6 cells per T175 cell culture flask and were passaged when they reached confluency using 0.25% Trypsin-EDTA solution. Wi38 cells were cultured in Eagle's minimal essential medium supplemented with 10% v/v FBS, 2 mM L-glutamine, 26 mM sodium bicarbonate, 100 U/ml penicillin/streptomycin and 1 mM sodium pyruvate; this was referred to as Wi38 medium. Cells were used between passages 18-24. Co-culture medium used was a 1:1 mixture of Calu-3:Wi38 medium. Unless otherwise stated, all cell culture and incubation steps were performed at 37°C and 5% CO₂ in a humidified atmosphere.

3.2.3.2 Polarised Epithelial Monoculture on Transwell® Inserts

For epithelial monocultures, Calu-3 epithelial cells were seeded into the apical side of 12 mm Transwell® inserts (VWR, Dublin, Ireland) as described in Section 2.2.3.3 (Figure 3.1). Briefly, 1 ml of co-culture medium was transferred onto the basolateral compartment and 400 µl into the apical compartment of the insert. The apical compartment of the Transwell® insert was then seeded with 5.65×10^5 Calu-3 cells in

100 μ l of Calu-3 medium and incubated for three days, which represents a seeding density of 5×10^5 cells/cm². At day three of culture, medium from the apical compartment was removed to introduce air-liquid interface (ALI) conditions. Cells on Transwell® inserts were fed every 2-3 days with 500 μ l of co-culture medium in the basolateral compartment.

3.2.3.3 Polarised Epithelial Co-culture on CHyA-B Scaffolds

After chemical cross-linking, CHyA-B scaffolds of 15.6 mm-diameter and 1 mm in height were preconditioned using 3 ml of co-culture medium and seeded according to Section 2.2.3.4 (Figure 3.1). For epithelial co-cultures on CHyA-B scaffolds, the porous layer of each scaffold was seeded with 6×10^5 Wi38 cells in 50 μ l of co-culture medium. Samples were incubated for 2 h to encourage cell attachment and supplemented with 3 ml of fresh medium thereafter. After two days of cell acclimatisation to the scaffold, they were pinned into the frame of a Snapwell® cell insert and seeded with Calu-3 cells. The apical side of the scaffold was seeded using 5.65×10^5 Calu-3 cells in 100 μ l of Calu-3 medium and incubated for three days, which represents a seeding density of 5×10^5 cells/cm². At day three of culture, medium from the apical compartment was removed to introduce ALI and samples were fed every 2-3 days with 2 ml of co-culture medium in the basolateral compartment.

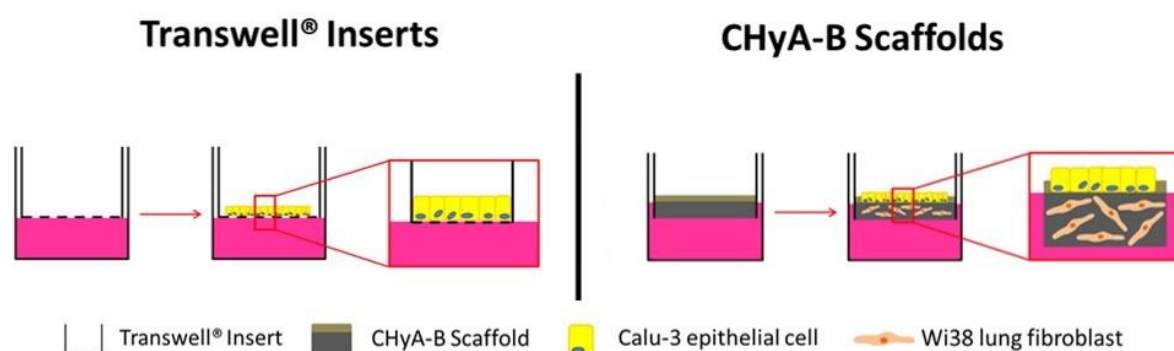


Figure 3. 1. Schematic of culture conditions used for Calu-3 epithelial monoculture on Transwell® inserts and Calu-3 Wi38 epithelial co-culture on CHyA-B scaffolds.

3.2.4 Evaluation of Epithelial Barrier Integrity

Transepithelial electrical resistance (TEER) values from epithelial cultures on Transwell® inserts and CHyA-B scaffolds were quantified throughout the cell culture

period to monitor epithelial barrier integrity. Fresh medium was added to the apical and basolateral compartments and TEER was measured using an EVOM voltohmmeter (World Precision Instruments, Hitchin, UK), STX-2 chopstick electrodes (World Precision Instruments) for Transwell® inserts and STX-3 chopstick electrodes (World Precision Instruments) for CHyA-B scaffolds. Transwell® inserts were filled with 500 µl of fresh co-culture medium in the apical and 1 ml in the basolateral compartment, while 500 µl were added to the apical side of CHyA-B scaffolds and 2 ml to the basolateral compartment. Electrical resistance on cultures was measured on day 3, 5, 8, 10, 12 and 14 of culture, and TEER values were calculated by subtracting the resistance of a cell-free scaffold or insert and corrected to the surface area (1.12 cm²) available for cell growth. Recovery of TEER following relevant exposure to nanoparticles and toxicants was determined as described in Section 2.2.5.1 using Equation 2.1.

3.2.5 *In vitro* Exposure

3.2.5.1 Nanoparticle Selection and Preparation

After 14 days in culture, epithelial cultures grown on Transwell® inserts and CHyA-B scaffolds were exposed for 24 h to a selection of commonly used nanomaterials (PLGA nanoparticles and CNTs) and novel nanomedicines (G5 miR and NC nanoparticles) developed in Prof. Cryan's research group to evaluate the effect of culture conditions on epithelial responses (Table 3.1). Lipopolysaccharide (LPS) and bleomycin from Chapter 2 were used as positive controls to evaluate toxicological and inflammatory epithelial responses. All exposure reagents were diluted to the required concentration using 1% v/v serum Calu-3 medium. LPS derived from *Pseudomonas aeruginosa* (*P.aeruginosa*) was used at a concentration of 10 µg/ml and bleomycin sulfate from *Streptomyces verticillus* at a concentration of 100 µg/ml in 1% v/v Calu-3 medium, as this was proven to be an optimal bacterial and drug-mediated challenge in Chapter 2. PLGA nanoparticles (200 nm average diameter) were used at a working concentration of 200 µg/ml in 1% v/v serum Calu-3 medium as previously established using non-polarised Calu-3 cultures⁴⁰⁷. Multi walled CNTs (110-170 nm in diameter, 5-9 µm in length) were used at concentration equal to 100 µg/ml, which has been described as a potential model of nanomaterial causing lung inflammation⁴⁰⁸. G5 star-shaped poly(L-lysine) polypeptides G5(64)PLL₅ (64-star-PLL) (G5 = generation 5 PPI

core, (64) = 64 polymeric L-lysine arms, $s = 5$ L-lysine subunits per arm, mol. weight = 94 000 g/mol) were synthesised and provided by the Polymer Chemistry and Biopolymer Labs in the RCSI University of Medicine and Health Sciences as previously described^{402, 409}. G5 polymers were complexed with miR-146a (G5 miR) or with a non-coding sequence (G5 NC) kindly synthesised and characterised by Rachel Gaul from the School of Pharmacy and Prof. Cryan's research group in RCSI University of Medicine and Health Sciences.

Table 3. 1. Size, charge and working concentration used for each of the nanoparticles use to expose epithelial cultures

Nanoparticle	Size	Charge/Zeta Potential	Working Concentration
PLGA	200nm (diameter)	$-35.5 \pm 12.4 \text{ mV}^{410}$ $-24 \pm 1 \text{ mV}^{411}$ $-5 \text{ mV to } -15 \text{ mV}^{412}$ -5 mV^{413}	200 $\mu\text{g/ml}$
CNTs	110-170nm (diameter) 5-9 μm (length)	Negative ^{414, 415}	100 $\mu\text{g/ml}$
G5 miR	$\approx 130 \text{ nm}^{416}$	$\approx 38 \text{ mV}^{416}$	3.13 $\mu\text{g/ml}$
G5 NC			3.87 $\mu\text{g/ml}$

3.2.5.2 Cell Toxicity

Following relevant exposure, cell toxicity on epithelial cultures was assessed at day 15 and 23 of culture (24 and 216 h after exposure) using alamarBlue™ Cell Viability Reagent (Biosciences, Dublin, Ireland) and Invitrogen™ LDH Cytotoxicity Assay (Fisher Scientific) to evaluate differences in epithelial responses between Transwell® and CHyA-B cultures. Apical and basolateral samples were first collected to evaluate lactate dehydrogenase (LDH) release, a colorimetric method to assess cellular toxicity. 50 μl of supernatant was pipetted into a Corning® 96-well Clear Flat Bottom Polystyrene Not Treated Microplate (Fisher Scientific) and incubated with 50 μl of Reaction Mixture, which was a combination of 600 μl of Assay Buffer Stock Solution with 11.4 ml of Substrate Stock Solution. Samples were incubated for 30 min at room temperature (RT) before adding 50 μl of Stop Solution. Absorbance was measured at 490 nm and 680 nm in a Tecan Infinite M Plex plate reader. LDH activity was obtained

by subtracting the 680 nm absorbance value from the 490 nm value, and normalised to the LDH activity from untreated cultures for each culture condition. An LDH positive control (Max LDH) was included for each culture condition consisting of epithelial cells treated with 10 µl of 10X Lysis Buffer for 45 min at 37°C prior to supernatant collection. LDH release values were expressed as change in release (%) relative to untreated cultures for each culture condition. Transwell® and CHyA-B cultures were incubated with 1X alamarBlue™ solution in co-culture medium using 500 µl for the apical and basolateral compartments of Transwell® cultures for 2 h. CHyA-B scaffolds were incubated with 500 µl and 2 ml of 1X alamarBlue™ solution in the apical and basolateral compartments respectively. After 2 h incubation at 37°C, 100 µl of supernatant were transferred to a Corning® 96-well Black Flat Bottom Polystyrene Not Treated Microplate (Fisher Scientific) protected from light. Fluorescence was measured using a 545 nm excitation and 590 nm emission wavelengths in a Tecan Infinite M Plex plate reader. Cell metabolic activity values were expressed as change in metabolic activity (%) relative to untreated cultures for each culture condition.

3.2.5.3 Secretion of Pro-inflammatory Cytokines

Potential inflammation in epithelial cultures arising after exposure was assessed by evaluating the secretion of interleukin-8 (IL-8), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) by enzyme-linked immunosorbent assay (ELISA) using ELISA MAX™ Deluxe Set IL-8, IL-6 and TNF- α (Biolegend, London, UK). Apical and basolateral samples were collected and centrifuged at 1,200 rpm for 5 min using a Heraeus' Biofuge Pico centrifuge before storage at -20°C prior to assay evaluation. A 96-well plate was coated overnight using 100 µl/well of the respective 200X Capture Antibody diluted 1:200 in 1X Coating Buffer. The plate was washed 4x between incubations using 0.05% v/v Tween-20 DPBS. Nonspecific binding was blocked for 1 h at RT using 200 µl/well of 1X Assay Diluent. 100 µl of sample was added to each well diluted appropriately (1:10 dilution for IL-8; no dilution used for IL-6 or TNF- α) and incubated for 2 h at RT. 100 µl of 200X Detection Antibody diluted 1:200 in 1X Assay Diluent was added to each well for 1 h incubation at RT followed by 30 min RT incubation with 100 µl/well of 1000X Avidin-HRP diluted 1:1000 in 1X Assay Diluent. Finally, each well was incubated with 100 µl of Substrate solution for 15 min and the reaction was stopped upon addition of 100 µl 2 N sulfuric acid (H₂SO₄). Absorbance was measured at 450 and 570 nm using a Tecan Infinite M Plex plate reader. Cytokine

release was calculated by subtracting the 570 nm value from the 450 nm and protein concentration estimated using standard calibration curve following manufacturer's instructions.

3.2.6 Data Analysis

Quantitative data obtained was analysed using Microsoft Excel and GraphPad Prism 7.0 Software. In cases of analysis between two groups, statistical difference was assessed by two-tailed Student t-test. For multiple groups, statistical difference between groups was assessed by 2-way Analysis of Variance (ANOVA). For analysis of the TEER measurements, repeated measures were performed as part of the 2-way ANOVA.

3.3 Results

3.3.1 Evaluation of Epithelial Barrier Integrity

Calu-3 epithelial monocultures were successfully established in Transwell® inserts with desired barrier properties as observed by TEER measurement (Figure 3.2) as previously reported (Chapter 2)¹³². This Transwell® epithelial monoculture is a commonly used model in the evaluation of inhaled nanoparticles and was selected as the 2D model^{357, 417}. Epithelial co-cultures of Calu-3 and Wi-38 cells were grown on CHyA-B scaffolds as the 3D model and they demonstrated comparable TEER values ($787.4 \pm 52.84 \Omega\text{cm}^2$) to Transwell® cultures ($708 \pm 67.92 \Omega\text{cm}^2$) with no significant differences between them at all time points. This data demonstrated the formation of a differentiated epithelial layer in both Transwell® and CHyA-B cultures to provide a realistic airway air-liquid interface to assess the effect of different nanoparticles.

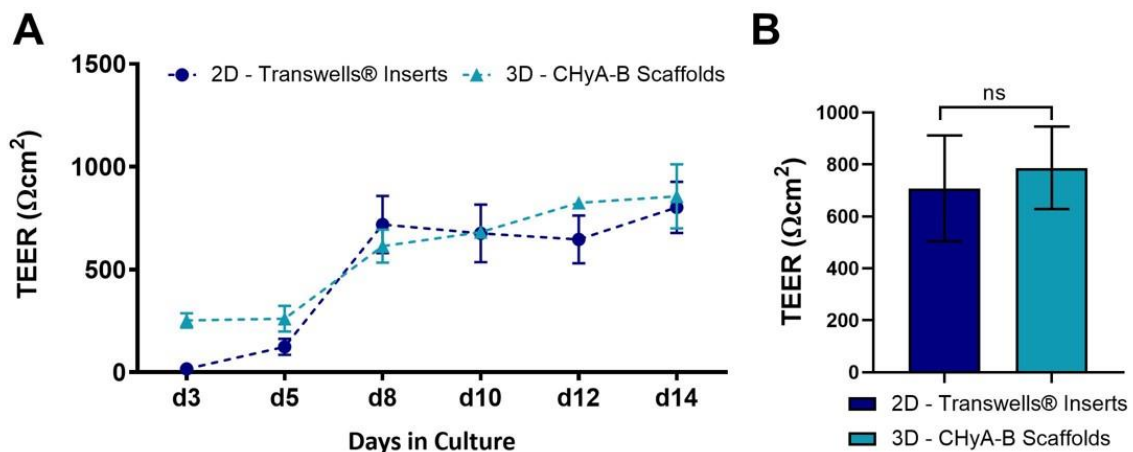


Figure 3. 2. Barrier integrity from epithelial monocultures grown on Transwell® inserts and epithelial co-cultures from CHyA-B scaffolds. (A) TEER values measured at day 3, 5, 8, 10, 12 and 14 from Calu-3 epithelial monocultures on Transwell® inserts (dark blue) and Calu-3 Wi-38 epithelial co-cultures on CHyA-B scaffolds under ALI conditions. (B) Average TEER values of epithelial cultures following plateau of electrical resistance measured at day 10, 12 and 14. Results displayed as mean $\pm$ SEM. (n=3).

3.3.2 Short-term Toxicological and Inflammatory Assessment of Nanoparticles

Four nanoparticles (PLGA, CNTs, G5 miR and G5 NC) were selected as examples of commonly used nanomaterials and novel nanomedicines used in drug delivery applications to evaluate epithelial responses following exposure between 2D and 3D. Following exposure of epithelial cultures to the four selected nanoparticles, cellular toxicity and inflammation were evaluated to investigate the short-term effects at day 15 in culture -24 h after exposure- and to detect different responses between Transwell® epithelial monocultures and CHyA-B epithelial co-cultures. For short-term assessment, LPS was used as a positive control as previously demonstrated in Chapter 2.

3.3.2.1 Cell Toxicity after Nanoparticle Exposure in 2D and 3D

Nanoparticle toxicity in epithelial cultures was assessed using alamarBlue™ and LDH release, biochemical assays commonly used to determine cell metabolic activity and cell toxicity from the apical and basolateral compartment of epithelial cells grown under ALI conditions. Overall, no significant differences in metabolic activity in comparison to untreated cultures were detected for PLGA nanoparticles (200 µg/ml), CNTs (100 µg/ml), G5 miR (3.13 µg/ml) and G5 NC (3.87 µg/ml) nanoparticles at the concentrations studied in both Transwell® and CHyA-B cultures when evaluated in the apical and basolateral compartment (Figure 3.3). Moreover, no significant differential response between Transwell® and CHyA-B cultures was observed for the nanoparticles employed for exposure. There was a very slight increase seen in metabolic activity for G5 NC treated cultures this was not statistically significant. LPS, used as a positive control for early-time induced toxicity, showed decreased metabolic activity in CHyA-B cultures compared to Transwell® cultures although this was only significant for the apical compartment. Bleomycin did not cause any cell toxicity 24 h after exposure which is in line with findings previously reported in Chapter 2 (Figure 3.3A,B).

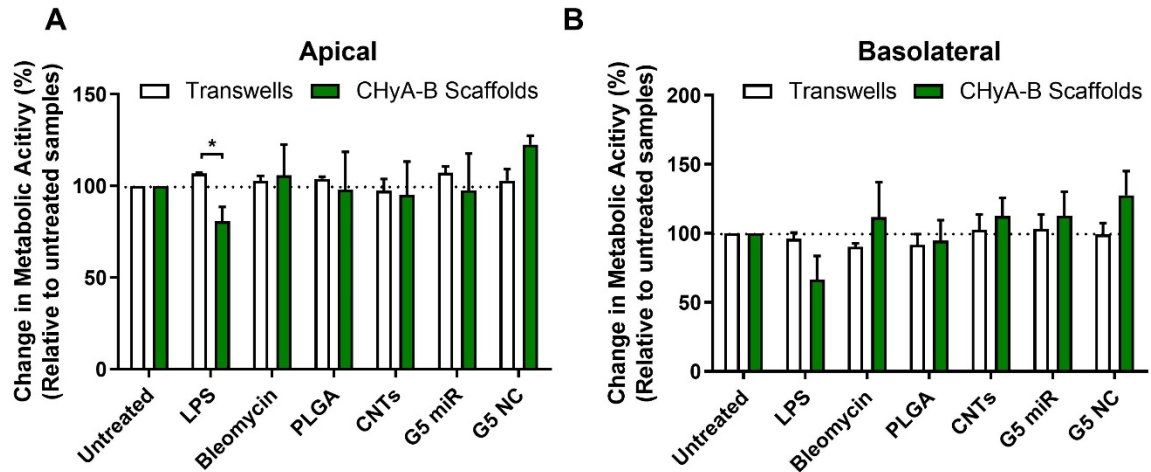


Figure 3. 3. Apical (A) and basolateral (B) cell metabolic activity measured from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Results displayed as mean $\pm$ SEM (n=3, *p<0.05).

Nanoparticle-mediated toxicity was further assessed by measuring the release of LDH from supernatants of epithelial cultures (Figure 3.4). No apical increase of LDH following exposure was detected in cultures grown on Transwell® inserts when compared to untreated cultures. However, a trend for higher LDH release in CHyA-B scaffolds was observed for all treated culture groups in comparison to untreated cultures. Moreover, when the apical LDH release for each condition was compared between culture conditions, a significant increase was detected in CHyA-B compared to Transwell® cultures for PLGA and G5 NC. Although a clear trend for higher LDH release into the apical compartment for all exposed conditions was visible, this was lower when compared to LDH release from positive controls (Max LDH) that were treated with lysis buffer prior to sample evaluation (Figure 3.4A). On the other hand, no significant differences were seen in the basolateral side of the Transwell® and CHyA-B cultures relative to untreated ones or between culture conditions. A small decrease in LDH release was observed for CNTs in CHyA-B versus Transwell® cultures but this was not statistically significant (Figure 3.4B).

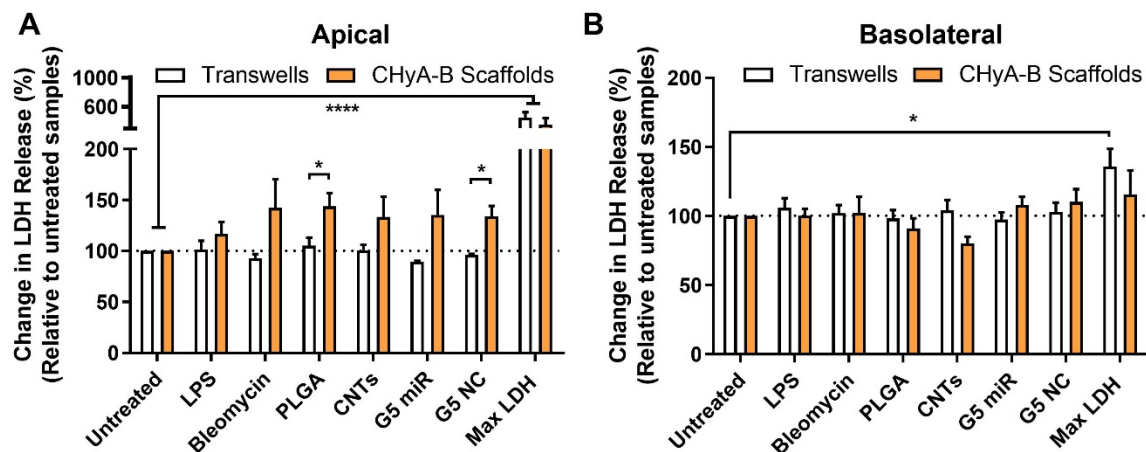


Figure 3. 4. Apical (A) and basolateral (B) LDH release from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Max LDH denotes epithelial cultures treated with Lysis Buffer acting as positive controls for LDH release. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, ***p<0.0001).

3.3.2.2 Inflammatory Response after Nanoparticle Exposure in 2D and 3D

The inflammatory profile of epithelial monocultures on Transwell® inserts and CHyA-B was studied to evaluate the effect of nanoparticles exposure and culture conditions on the secretion of IL-8 and IL-6 pro-inflammatory cytokines, key mediators of airway epithelium inflammation ⁴¹⁸. The release of these cytokines was measured in both the apical and basolateral compartments following treatment.

A differential IL-8 secretion profile was seen after exposure between Transwell® and CHyA-B scaffolds cultures. Apical IL-8 levels from all treated groups were comparable to untreated cultures with the exception of LPS in Transwell® cultures, which has previously been demonstrated to increase IL-8 secretion 24 h after exposure (Chapter 2). However, there was an increase, though not statistically significant, in IL-8 expression from CHyA-B cultures after treatment with CNTs, and G5 NC in comparison to untreated, which was not seen in Transwell® cultures. There was a significant increase in secretion of IL-8 in CHyA-B cultures treated with LPS and G5 miR treated cultures (Figure 3.5A). LPS was the only treatment to show an increase (though not significant) in IL-8 expression in Transwell® insert cultures in comparison to untreated. The basolateral secretion of IL-8 showed slight increases in CNTs, G5 miR and G5 NC when compared to the relevant untreated condition. IL-8 levels in cultures treated with LPS and G5 miR were significantly higher in CHyA-B in comparison to Transwell® epithelial cultures (Figure 3.5B). Although IL-8 secretion was increased in CHyA-B treated with LPS and G5 miR nanoparticles, this was not significant compared to untreated cultures on CHyA-B scaffolds.

There were also differences seen in IL-6 secretion after nanoparticle treatment between Transwell® and CHyA-B cultures (Figure 3.6). Apical IL-6 expression from Transwell® cultures remained unchanged after nanoparticle treatment with the exception of LPS, which showed a significant increase (Figure 3.6A). Higher apical IL-6 levels across all groups between culture conditions (Transwell® versus CHyA-B cultures) were seen, albeit no significant increases were seen in CHyA-B cultures after treatment compared to untreated (Figure 3.6A). There was no increase in basolateral IL-6 levels in treated Transwell® cultures, with a trend for higher secretion after LPS exposure compared to untreated cultures. Secretion of IL-6 from CHyA-B cultures in all treated groups was similar to untreated controls (Figure 3.6B).

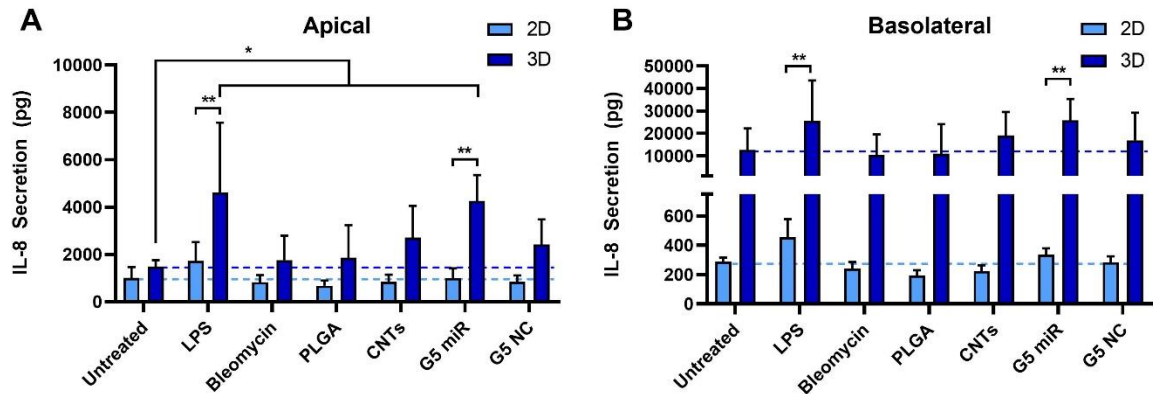


Figure 3. 5. Apical (A) and basolateral (B) IL-8 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-8 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, **p<0.01).

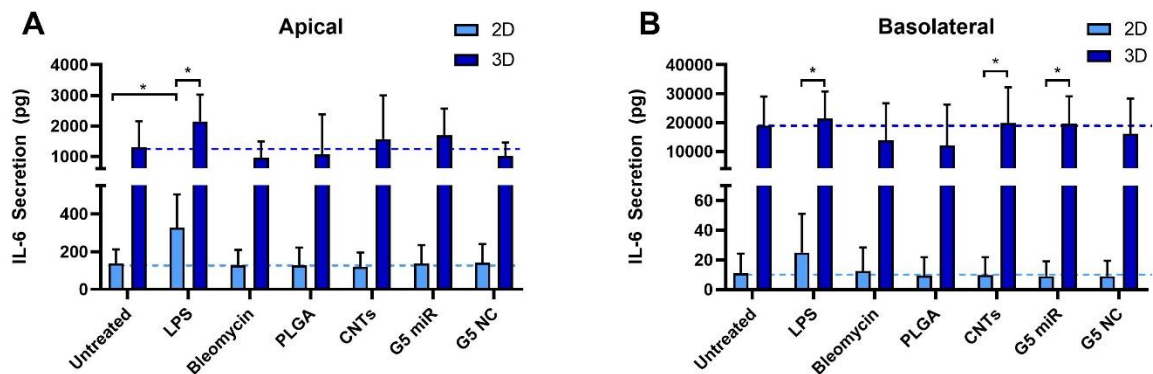


Figure 3. 6. Apical (A) and basolateral (B) IL-6 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 15 of culture, 24 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-6 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05).

3.3.3 Long-term Toxicological and Inflammatory Assessment of Nanoparticles

Epithelial Transwell® and CHyA-B cultures were exposed to the selected nanoparticles at day 14 of culture as described in Section 3.3.2. For long-term assessment, cellular toxicity and inflammation were evaluated at day 23 of culture, 216 h (9 days) after relevant exposure to detect any possible differences between Transwell® epithelial monocultures and CHyA-B epithelial co-cultures. For long-term assessment, bleomycin was used as a positive control as previously outlined in Chapter 2.

3.3.3.1 Cell Toxicity after Nanoparticle Exposure in 2D and 3D

Cell toxicity after nanoparticle treatments was evaluated using alamarBlue™ and LDH assays (Figure 3.7, Figure 3.8). There were no differences in apical cell metabolic activity in Transwell® and CHyA-B cultures after nanoparticle treatment compared to untreated samples. Only bleomycin resulted in decreased cell metabolic activity in CHyA-B cultures compared to Transwell® cultures. There were no significant differences in metabolic activity between Transwell® CHyA-B scaffolds for the other exposure conditions (LPS, PLGA, CNTs, G5 miR and G5 NC) (Figure 3.7A).

Similarly, exposure of epithelial cultures did not lead to changes in basolateral cell metabolic activity across all treated groups in Transwell® cultures, with the exception of a trend for decreased activity in bleomycin-treated cultures. However, CHyA-B cultures showed a trend towards higher metabolic activity after bleomycin, PLGA, CNTs, G5 miR and G5 NC treatment compared to untreated cultures. Moreover, a significant difference in cell metabolic activity between Transwell® and CHyA-B cultures was detected after G5 NC exposure, which was not seen in any other exposure condition (Figure 3.7B). Nanoparticle-mediated toxicity in epithelial Transwell® and CHyA-B cultures was also evaluated by determining the release of LDH (Figure 3.8). There were no significant differences between apical responses from treated Transwell® and CHyA-B cultures in comparison to untreated controls, with the exception of bleomycin. When comparing treatment responses between Transwell® and CHyA-B cultures, there were no significant differences (Figure 3.8A). This behaviour was also seen when evaluating the release of LDH into the basolateral compartment (Figure 3.8B).

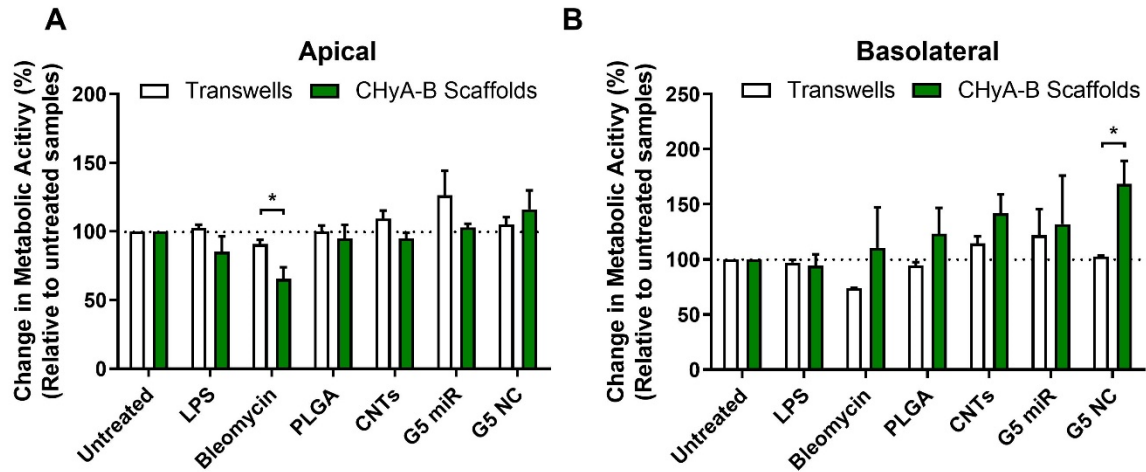


Figure 3. 7. Apical (A) and basolateral (B) cell metabolic activity measured from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 23 of culture, 216 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Results displayed as mean $\pm$ SEM (n=3, *p<0.05).

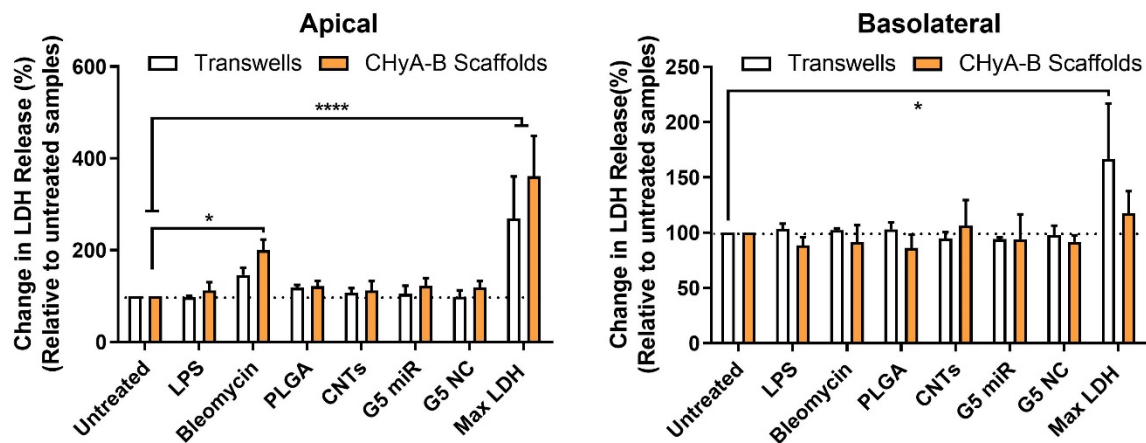


Figure 3. 8. Apical (A) and basolateral (B) LDH release from Calu-3 epithelial cultures on Transwell® inserts and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds measured at day 23 of culture, 216 h after exposure. Max LDH refers to epithelial cultures treated with Lysis Buffer acting as positive controls for LDH release. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, ****p<0.0001).

3.3.3.2 Inflammatory Response after Nanoparticle Exposure in 2D and 3D

Epithelial cultures were exposed to nanoparticles at day 14 of culture and long-term induced inflammatory responses were assessed at day 23 of culture, 216 h (9 days) after exposure. IL-8 and IL-6 levels in the apical and basolateral compartments from epithelial Transwell® monocultures and CHyA-B co-cultures were examined (Figure 3.9, Figure 3.10). Only bleomycin lead to a significantly higher apical IL-8 secretion from Transwell® cultures compared to untreated cultures. On CHyA-B scaffolds, a significant increase in bleomycin-treated cultures compared to untreated cultures was also observed. A trend for higher apical IL-8 release was observed for the G5 miR and G5 NC nanoparticles, although this was not significant. When comparing cellular inflammatory responses between culture conditions, a significantly higher increase in IL-8 was again detected for bleomycin treated CHyA-B versus Transwell® cultures. A higher secretion of IL-8 from CHyA-B cultures was seen after treatment with LPS, G5 miR and G5 NC compared to Transwell® cultures but was not significant (Figure 3.9A). Basolateral detection of IL-8 in CHyA-B cultures in comparison to Transwell® cultures was significantly increased for all culture conditions with the exception of PLGA nanoparticles (Figure 3.9B).

To further investigate the effect of treatments on cell responses, the release of IL-6 into the apical and basolateral compartments was investigated (Figure 3.10). Transwell® cultures exposed to nanoparticles did not show significant differences in apical IL-6 secretion in comparison to untreated cultures, with the exception of a non-significant increase for the positive controls LPS and bleomycin. The same behaviour was observed in CHyA-B cultures but showing a trend for increased secretion for G5 miR in comparison to untreated cultures. Overall, higher levels of IL-6 were detected in the apical compartment of CHyA-B in comparison to Transwell® cultures, but this showed significance only for cultures exposed to G5 miR nanoparticles (Figure 3.10A). In the basolateral compartment, IL-6 levels from Transwell® cultures were higher for all exposed groups in comparison to untreated samples although this was only significant for bleomycin. For CHyA-B cultures, no significant increase in IL-6 secretion was found after nanoparticle treatment compared to untreated cultures. A significant increase in IL-6 secretion was seen after CHyA-B cultures were treated with G5 miR nanoparticles as well as the positive controls, LPS and bleomycin. Overall, IL-6 levels

were observed to be higher in CHyA-B compared to Transwell® cultures (Figure 3.10B).

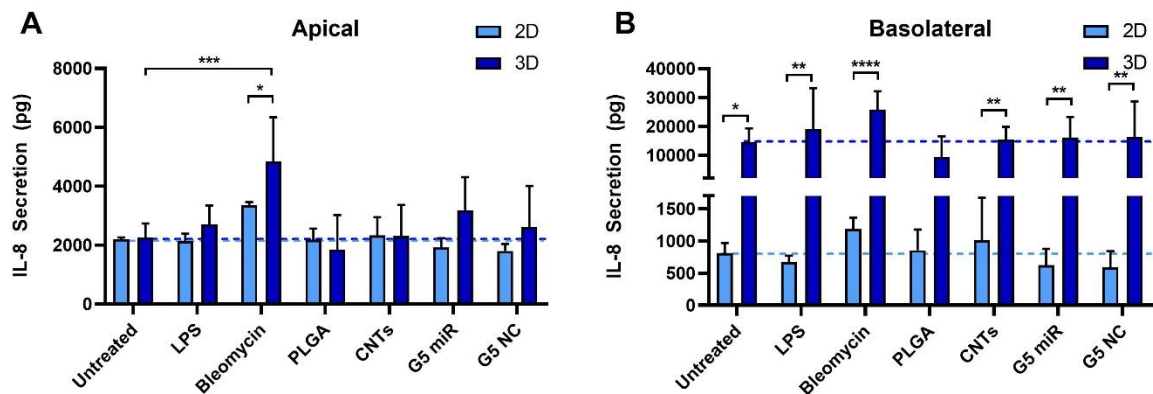


Figure 3. 9. Apical (A) and basolateral (B) IL-8 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 23 of culture, 216 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-8 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

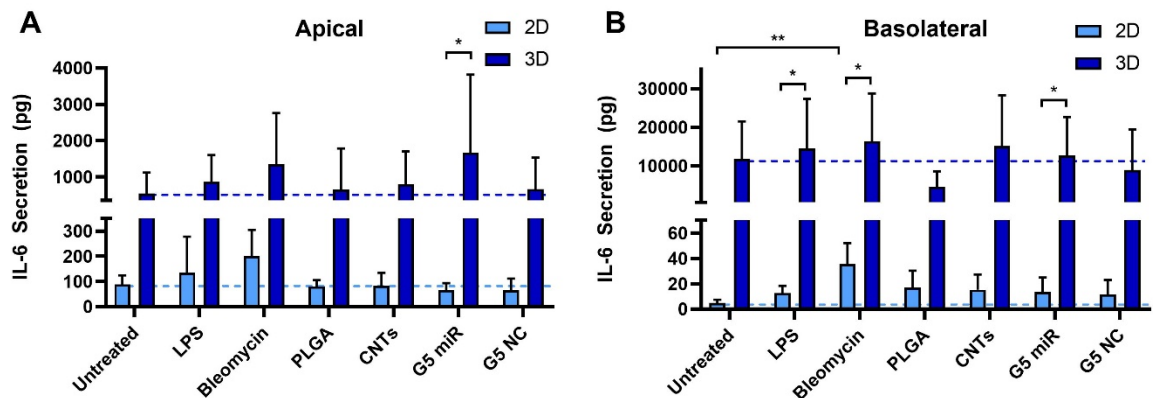


Figure 3. 10. Apical (A) and basolateral (B) IL-6 secretion from Calu-3 epithelial monocultures on Transwell® inserts (2D) and Calu-3 Wi38 epithelial co-cultures on CHyA-B scaffolds (3D) measured at day 23 of culture, 216 h after exposure to LPS, bleomycin, PLGA nanoparticles, CNTs, G5 miR and G5 NC nanoparticles. Dotted light and dark blue line denotes basal IL-6 secretion for untreated cultures on Transwell® inserts and CHyA-B scaffolds respectively. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, **p<0.01).

3.3.3.3 Barrier Integrity

The recovery of epithelial barrier integrity estimated by measuring TEER values across Transwell® and CHyA-B cultures was used to investigate the effect of nanoparticle exposure in the maintenance of an epithelial monolayer as well as to elucidate differential cell behaviors across the two different culture conditions. Overall, a sufficient TEER recovery (close to 100% recovery relative to untreated cultures) was observed for Transwell® and CHyA-B cultures on day 23 of culture -216 h (9 days) post exposure-. A trend for decreased recovery in CHyA-B cultures at early time points compared to Transwell® cultures was observed (Figure 3.11). LPS exposure led to a significant decrease in TEER recovery at day 17 in CHyA-B cultures in comparison to Transwell® ones. Bleomycin-treated cultures in CHyA-B scaffolds showed a significant decrease in comparison to Transwell® cultures on day 19 and 21 (Figure 3.11A,B). Exposure to PLGA nanoparticles resulted in a decreased recovery in CHyA-B on day 19 following exposure compared to Transwell® cultures (Figure 3.11C). For CNT-treated cultures, a decreased recovery in CHyA-B versus Transwell® cultures was observed on day 17 and 19 of culture (Figure 3.11D). TEER recovery following exposure to G5 miR nanoparticles did not differ significantly between CHyA-B and Transwell® cultures (Figure 3.11E). Exposure to G5 NC nanoparticles led to a lower recovery on day 19 in CHyA-B compared to Transwell® cultures (Figure 3.11F).

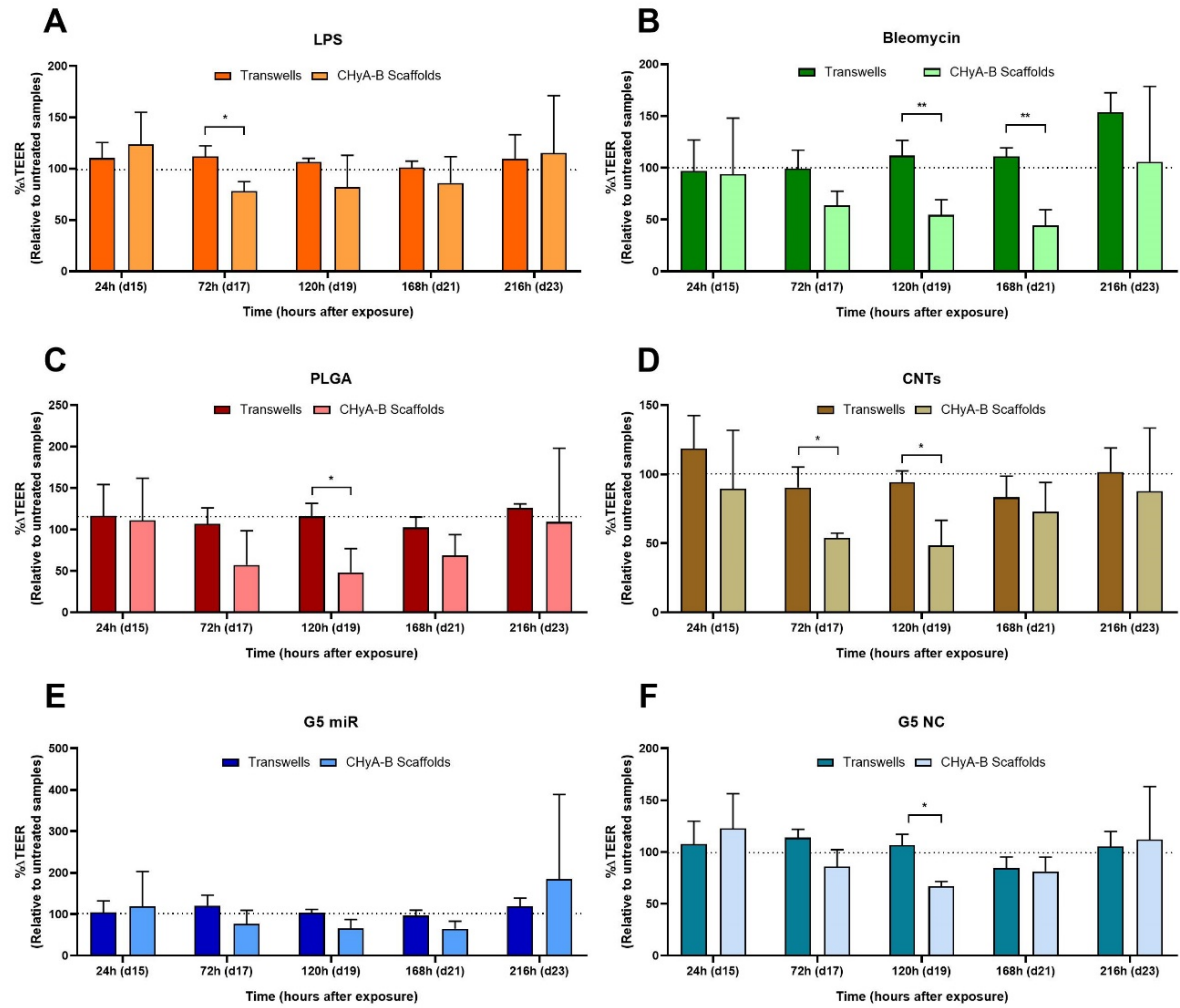


Figure 3. 11. TEER recovery of epithelial Transwell® monocultures (light) and epithelial CHyA-B co-cultures (dark) following exposure to LPS (A), bleomycin (B), PLGA nanoparticles (C), CNTs (D), G5 miR (E) and G5 NC (F) nanoparticles measured at day 15, 17, 19, 21 and 23 of culture following exposure. TEER recovery values were normalised to the recovery of untreated samples for each culture condition. Results displayed as mean $\pm$ SEM ($n=3$; * $p<0.05$, ** $p<0.01$).

3.4 **Discussion**

The development of novel *in vitro* models of the airway has increased the understanding of the complex airway epithelial responses against inhaled nanoparticles, toxicants or pathogens⁴¹⁹. The development of these airway models can overcome limitations posed by conventional animal testing by allowing for high-throughput analysis of multiple compounds simultaneously, reducing associated cost as well as eliminating ethical concerns raised by animal testing^{351, 420}. Primary cells e.g. NHBEs can be used to improve *in vitro* airway models but their cost, tedious subculture, differentiation protocols and variability between donors makes bronchial cell lines a more favourable choice³⁵⁰. Among several bronchial cell lines commercially available that allow for cheap, well-characterised models, Calu-3 cells are the current gold standard due to their ability to form tight junctions and a mucus layer²³⁰. Interestingly, Calu-3 secretome has been recently deeply characterised showing abundant similarities to NHBE-derived epithelium. All taken together, the Calu-3 cell line has proved to be a promising alternative to the use of primary cells allowing for the development of robust and cost-effective models as hereby presented.

The TEER measurements from epithelial co-cultures in CHyA-B scaffolds (Figure 3.4A) matched those reported in Chapter 2 and literature, demonstrating the formation of a polarised airway epithelium which is crucial to replicate *in vivo* physiological conditions^{132, 421, 420}. The formation of a mature and functional epithelial barrier is essential for drug screening, as the formation of tight junctions and cell polarisation regulating transcellular flux have been proven to affect drug uptake⁴²². Mimicking epithelial barriers and understanding the mechanism behind regulation of transport across airway epithelial barriers is essential to develop better testing platforms for novel nanotherapies²⁴⁴. Here, we again demonstrated that these features are replicated using epithelial co-cultures in CHyA-B scaffolds, making it an alternative model to understand and identify potential toxic effects of novel therapies. Furthermore, we monitored the changes in epithelial barrier integrity after exposure to selected nanoparticles.

Reduction in TEER values across epithelial barriers is hypothesised to arise from tight junction damage which leads to barrier impairment, but does not necessarily arise from cell death⁴²³. Long-term evaluation of epithelium integrity is crucial to assess potential

side effects that can go unnoticed in 2D conventional cultures but can be detected using 3D cultures. Bleomycin treatment was used as a positive control for long-term epithelial toxicity, cell death and decreased barrier integrity with lower TEER recovery in CHyA-B cultures on day 19 and 21 of culture compared to Transwell® cultures. There was good recovery in epithelial barrier integrity in Transwell® cultures after nanoparticle exposure, but it was more limited for CHyA-B cultures (Figure 3.11). Interestingly, CNT treatment led to a reduced barrier recovery rate in CHyA-B when compared to Transwell® cultures, but no toxicity or cell death were observed (Figure 3.7, Figure 3.8). This matches previous findings showing no apparent induced toxicity on alveolar models and a transient alteration of barrier integrity which then returned to pre-exposure TEER values⁴²³. Other studies investigating the effect of CNTs on Calu-3 monolayers also reported reductions in TEER measurements but cell viability remained unaffected after exposure⁴²⁴. Polarised Calu-3 cultures exposed to CNTs concentrations ranging from 4 µg/cm² – 4 pg/cm² also resulted in decreased TEER measurements, although this was only investigated 24 and 48 h post-treatment and barrier recovery could not be assessed⁴²⁵. No apparent impairment of barrier function was detected after exposure to G5 miR nanoparticles, although a significant decrease was observed following exposure to PLGA and G5 NC nanoparticles in CHyA-B cultures at day 19 after exposure (Figure 3.11). The lower TEER values after exposure from CHyA-B cultures can be explained by recent reports showing reduced TEER measurements in 3D *in vitro* models. This has been hypothesised to arise from changes in porosity between 2D and 3D models as well as the formation of less strong tight junctions using 3D models of the gastrointestinal epithelium⁴²⁶. The use of epithelial 3D models could provide more accurate barrier function when compared to *in vivo* responses, allowing for better understanding of nanoparticle exposure and its effect in barrier integrity.

In Chapter 2, the inclusion of a 3D environment and epithelial co-culture with lung fibroblasts was shown to modulate cell responses. Upon exposure of airway cells to LPS and bleomycin, substances known to cause airway inflammation and cell toxicity^{334, 362, 364, 411, 427}, differential responses were observed when cells were grown on CHyA-B scaffolds compared to conventional 2D models (Transwell® inserts) commonly used in drug screening and development. This TE *in vitro* co-culture model was further challenged to evaluate its performance upon exposure to common

nanomaterials and novel nanomedicines. Epithelial toxicity and the secretion of key pro-inflammatory mediators for cells cultured on CHyA-B scaffolds were examined in comparison to epithelial monocultures on Transwell® inserts while using LPS and bleomycin as positive controls for cellular responses. Ultimately, we aimed to investigate whether collagen-based scaffolds can be employed as a robust *in vitro* model to detect any adverse effect from inhaled nanotherapies. Thus, enabling for the detection of epithelial responses that can go unnoticed when using 2D models of the airway for drug efficacy and routine safety testing.

Short-term toxicity and inflammatory effects of inhaled substances has been previously performed using 2D models consisting of Calu-3 epithelial cultures^{428, 429}. However, single cell 2D models do not always effectively predict the toxic *in vivo* response as the lack of an ECM-like environment and co-culture conditions might not fully reflect epithelial responses⁴³⁰. No significant short-term toxicity after assessing cellular metabolic activity and LDH release was seen in either the basolateral or apical compartment in either Transwell® or CHyA-B cultures, apart from a significantly reduced apical metabolic activity in CHyA-B scaffolds treated with LPS (10 µg/ml) (Figure 3.3). Interestingly, this LPS effect was not seen in Transwell® cultures, although LPS has been shown to induce alveolar epithelial cell death due to the production of reactive oxygen species when used at concentrations higher than 15 µg/ml⁴³¹. Nanoparticle exposure led to higher apical LDH release from CHyA-B compared to Transwell® cultures, although this was significantly lower compared to cultures treated with lysis buffer, the positive control used in the LDH assay. LDH secretion was significantly increased after G5 miR and PLGA nanoparticle treatment, positively and negatively charged nanoparticles respectively (Figure 3.4). Nanoparticle charge is of crucial interest when evaluating cellular toxicity following exposure. For instance, cationic formulations have been described to induce oxidative stress leading to cell damage while other studies have demonstrated toxicity in primary human airway epithelial cells upon exposure to positively charged nanomedicines^{427, 432-434}. We hypothesise that short-term cellular toxicity following inhaled therapeutic exposure can be evaluated using the apical compartment of this TE model. Here, we show toxicological responses to several nanomedicines differed between classic 2D culture and novel co-culture 3D TE models and the importance of the apical compartment in detecting these changes.

An apical increase in IL-8 was only detected for LPS treated Transwell® cultures. In CHyA-B cultures a significant increase in IL-8 secretion was seen after CNTs and both G5 miR nanoparticle treatment relative to untreated cultures (Figure 3.5A). This was also seen in the basolateral compartment of CHyA-B cultures treated with G5 miR nanoparticles, but not in Transwell® cultures (Figure 3.5B). As previously reported, neither PLGA nor CNTs treatment led to a significant apical or basolateral increase of IL-8 from Transwell® cultures^{407, 425}. The inclusion of 3D environments and co-culture conditions has already been described to produce differential inflammatory profiles after exposure when compared to 2D models. For example, 3D lung microtissues combining epithelial cells, fibroblasts and macrophages exposed to CNTs (0.5-10 µg/ml) led to higher expression of genes involved in inflammation including inflammatory cytokines, ECM proteins and matrix metalloproteases²⁵³. Multicellular 3D models allow for better recapitulation of paracrine signaling as well as cell-to-cell contacts, which provides a more realistic overview of cellular processes following inhalation. For instance, alveolar models of the airway comprising A549 cells, DC and macrophages showed a different pro-inflammatory (IL-6 and IL-8) secretion after exposure to LPS and TNF-α in comparison to single cell models⁴³⁵. In our study, differential inflammatory responses were seen in CHyA-B cultures in comparison to conventional 2D cultures after nanoparticle treatment, highlighting the need to assess inflammatory responses in more complex models to detect any increase of cytokine production that cannot not be detected when using simple Transwell® monocultures of the airway epithelium. Specifically, 3D models are particularly important to evaluate the complex crosstalk in inflammation following inhalation as epithelial cells play a crucial role in the activation and recruitment of cells²²⁶.

Short-term screening of acute toxicity and inflammation after treatments are often the focus, as long-term establishment of cell-line based cultures is hindered by decreased viability of the models over time as well as alterations of cellular characteristics⁴²⁰. In Chapter 2, we demonstrated the viability of epithelial cultures on CHyA-B scaffolds up to 23 days maintaining a differentiated epithelium supported by optimal TEER values after introduction of ALI conditions. In this chapter, we further demonstrated the maintenance of barrier integrity for longer-term studies of toxicity and inflammation up to 23 days of culture (Figure 3.11). Overall, no long-term toxicological effect on exposed cultures was seen over time for any of the nanoparticles tested albeit a trend

for increased basolateral metabolic activity in CHyA-B cultures compared to Transwell® cultures was observed, which was only significant for cultures treated G5 NC nanoparticles. The only toxic effect was detected for bleomycin-exposed cultures, the positive control optimised in Chapter 2 (Figure 3.7A, Figure 3.8A). Empty 200 nm in diameter PLGA nanoparticles have already been shown to not induce cellular toxicity and apoptosis in A549 and BEAS-2B airway cells⁴¹³. Although CNTs 50 nm in diameter are believed to cause cytotoxicity and carcinogenicity, thick CNTs 150 nm in diameter have proved to be less toxic, inflammogenic and carcinogenic, which matches the findings herein reported after exposure to CNTs 110-170 nm in diameter^{436, 437}. Here, CNTs 50 nm were chosen due to its higher cytotoxicity over 150nm to better evaluate cellular responses and prove the co-culture model as a tool to detect toxicity and inflammation. However, inclusion of further CNTs with different sizes and diameters should be explored to elucidate differences in epithelial responses. G5 miR and NC nanoparticles did not show any cytotoxic effect, which correlates with previous findings demonstrating their biocompatibility properties in different drug-delivery applications^{402, 404}.

This model also allows for the detection of longer-term induced inflammation after exposure, to provide valuable insights on nanoparticulate technology effects as it has been reported novel nanoparticles can accumulate over time leading to cellular adverse effects. Usually, studies looking at the inflammatory response of nanoparticles only evaluate early time points post-exposure thereby, overlooking the potential for long-term effects after inhalation of nanoparticles⁴³⁸. Apical release of IL-8 was increased after exposure to bleomycin at day 23 in culture in both Transwell® and CHyA-B cultures compared to untreated cultures (Figure 3.9A). Although there was no increase in secretion in Transwell® cultures for all treated conditions, a trend for increased secretion in CHyA-B cultures was observed after G5 miR exposure, which was not evident in Transwell®. Moreover, a differential profile with increased basolateral secretion of IL-8 was observed in CHyA-B cultures compared to their Transwell® counterpart (Figure 3.9B). A similar behaviour was also observed with the secretion of IL-6, but no exposed nanoparticle lead to a significant increase in pro-inflammatory cytokines when compared to untreated cultures (Figure 3.10). The lack of a longer-term inflammation after PLGA exposure correlates with findings showing no inflammatory responses up to 21 days after exposure in rat models and no increase

in the secretion of IL-8 and IL-6 after exposure using Calu-3 and A549 cells^{411, 439, 440}. CNTs have also not been reported to elicit an inflammatory response in mice models 21 days after initial exposure⁴⁴¹. Although contradictory data can be found regarding the inflammatory effect of inhaled CNTs, it has also been reported that the levels of IL-6 were not affected after a single exposure to CNTs evaluated more than 24 h post-exposure^{442, 443}. On the other hand, the lack of an increase in the secretion of pro-inflammatory markers after exposure to G5 miR nanoparticles corresponds with previous findings of this nanoparticles for cystic fibrosis research⁴¹⁶.

The introduction of a 3D environment and co-culture conditions in this model has increased the secretion of IL-8 and IL-6 from untreated and nanoparticle exposed cultures, with the exception of apical secretion of IL-8. This is evident for both apical and basolateral secretion of IL-6 when evaluating the short and long-term inflammatory cellular responses. This is hypothesised to arise from the inclusion of a fibroblast layer in the porous scaffold which is not present in Transwell® as previously shown⁴⁴⁴. IL-6 is believed to play a critical role in ECM-remodeling by lung fibroblasts and pulmonary fibrosis, and therefore, monitoring the secretion of this cytokine allows for better understanding of cellular responses in tissues underlying the airway epithelium⁴⁴⁵. Thus, this 3D model also opens the possibility to not only study the effect of inhaled therapies in the airway epithelium but also in the supporting of underlying tissues and how this translates into cytokine secretion in the apical compartment.

The latest trends in *in vitro* modelling for assessing the effect of novel nanoparticle formulations focus significantly on the use of 3D supported culture systems⁴⁴⁶. As presented here, the inclusion of a 3D ECM-like environment has the potential to allow identification of cell effects that can go unnoticed in simple planar 2D models. The culture of cells in 3D has been shown to affect cell proliferation and to offer higher cell-to-cell interaction which can result in different toxicity between 2D and 3D models³¹. For example, cadmium telluride nanoparticles showed lower toxicity in 3D spheroid cultures versus the standard 2D culture inducing a different type of cell death, presumably due to lower access of nutrients and toxicants in 3D⁴⁴⁷. On the other hand, exposure of A549 alveolar cells to ZnO nanoparticles showed higher toxicity in a 3D spheroid model⁴⁴⁸. An important aspect to study the effects of novel nanoparticulate technologies in cells grown on 3D matrices is the concentration used for exposure,

which has been shown to elicit different cellular responses. For instance, higher death and inflammation were seen in 2D compared to 3D for high concentrations but an opposite effect when using low concentrations after exposing Caco-2 cells grown on agarose gels and in 2D to ZnO nanoparticles³⁶⁹. Overall, 3D models commonly detect lower cytotoxicity post-exposure to nanoparticles, but they can be promising tools for the detection of cellular responses due to their increased sensitivity when compared to conventional models³¹. We introduced lung-derived fibroblasts in the porous sublayer of the CHyA-B scaffolds to support the epithelial cells and observed significant differences in cytokine release when compared to the Transwell® model. A different nanoparticle uptake profile has been observed in alveolar cells grown in the presence of immune and endothelial cells compared to monoculture systems, highlighting how uptake can be guided by cellular interactions between cell types³⁹⁵. The sensitivity of an *in vitro* model can be improved with co-culture set-ups, for instance, increased detection of IL-8 and IL-6 was observed in co-culture models after exposure to silica nanoparticles. When alveolar epithelial cells were grown in the presence of endothelial cells to mimic the alveolar-capillary barrier and were exposed to silica nanoparticles, increased cytokine secretion was observed in the basolateral compartment with more sensitive cellular responses compared to conventional monocultures³⁹⁶. The use of co-culture models holds promise in detecting adverse immune effects that otherwise are not pronounced and therefore, easily detected in 2D *in vitro* models³¹.

One main limitation of this model is the lack of an underlying immune cell type co-culture combined with the epithelial and fibroblast cells. Inclusion of commonly used macrophages (e.g. THP-1) and dendritic cells would allow the model to recapitulate inflammatory responses and cell crosstalk in a more complex manner. Thus, providing valuable insights on the dynamics of nanoparticle interaction with immune cell types found in the airway epithelium, as herein it has already been shown that the inclusion of other cell types e.g. fibroblasts has a clear effect in the pro-inflammatory cytokine secretion after exposure³⁴⁶. When using 3D scaffold systems for toxicological and inflammatory studies, it is of crucial importance to assess the effect that the artificial barrier creates by the chosen scaffold and how this might affect nanoparticle translocation and transport between tissue layers by the inclusion of appropriate controls e.g. blank scaffold. Herein, the importance of the apical compartment for

evaluating different cellular responses between 2D and 3D was reported. However, most exposure studies for inhaled formulations are carried out using aerosol set ups, which do not allow for evaluation of the apical compartment, where the liquid mucus layer is found. Finally, the route of administration for the nanoparticle under evaluation should be accurately represented during the *in vitro* safety testing. For example, most inhaled nanomedicines will be delivered using an inhaler device for aerosol exposure, which was not used in this study. This could represent a next step to further improve the model by using nebulisers, gas generators, microsprayers or commercially available exposure chambers such as Cultex® and Vitrocell®¹⁹. It is crucial to evaluate cellular responses on the apical compartment, as apical receptors are responsible for the activation of several signaling pathways⁷³. Overall, this Chapter has shown the feasible application of CHyA-B scaffolds containing airway epithelial and fibroblast cells as a novel *in vitro* model to assess the toxicological and inflammatory cellular responses following exposure.

3.5 Conclusion

Previously, in this thesis a co-culture *in vitro* model of the airway that provides physiologically relevant epithelial toxicological and inflammatory responses after exposure to external stimuli was validated. In this Chapter, further work was added by challenging the model with a range of nanoparticles in order to demonstrate its feasibility for *in vitro* screening of nanoparticles leading to the following conclusions:

- CHyA-B scaffolds show different epithelial barrier integrity after exposure to nanoparticles in comparison to Transwell® models, and can be therefore used to better monitor the barrier properties following nanoparticles exposure.
- Adverse toxicological and inflammatory responses to inhaled substances can be detected early using this 3D TE co-culture *in vitro* model. Otherwise, this might be unnoticed in 2D filter cultures, which might led to increased failure during clinical assessment.
- This model allows for both short (1 day) and longer-term (9 days) evaluation of epithelial cell responses after exposure in 3D co-culture conditions better mimicking the *in vivo* physiological conditions.

Thus at the end of this Chapter it can be concluded that the epithelial co-culture *in vitro* system on CHyA-B scaffolds can be easily applied to study the effects of novel therapies during their pre-clinical assessment in a more physiologically-relevant manner. Furthermore, this model could be easily upgraded by including other relevant cell types as well as by using the right exposure technology.

Chapter 4

Development, Characterisation and Biocompatibility of Polyglycolide 3D Printed Scaffolds for Tissue Engineering Applications

4.1 Introduction

Tissue-engineered (TE) techniques can be harnessed as research tools to better understand disease pathophysiology, develop novel treatments and cell-based therapies for the treatment of severe airway diseases. Moreover, they can be used to evaluate the safety and efficacy profile of potential treatments as demonstrated in Chapters 2 and 3⁴⁴⁹⁻⁴⁵¹. In addition, TE strategies can be harnessed in respiratory medicine to develop medical devices with regenerative properties to fully restore damaged airway tissue⁴⁵². One application is in tracheal tissue engineering, a field of respiratory regenerative medicine that focuses on offering a functional solution to tracheal defects caused by several types of cancers, inflammatory stenosis, infection or congenital abnormalities^{35, 255, 453}.

Several materials have been investigated in recent years with the objective of producing a functional tracheal substitute that can be easily implanted and support the formation of tracheal tissues including respiratory epithelium, cartilage and the formation of a vasculature network to maintain the device patency^{35, 267, 454, 455}. Both natural and synthetic materials have been investigated for tracheal replacement and general TE applications with the shared characteristics of being biocompatible, non-toxic products of degradation and non-carcinogenic⁴⁵⁶. Natural materials including collagen, hyaluronic acid (HyA), silk fibroin and elastin have been investigated for the development of tracheal scaffolds with promising results⁴⁵⁷. For instance, porous silk fibroin matrices have been shown to promote tracheal epithelial regeneration and elastin-rich decellularised pig tracheas have been described as robust extracellular matrix (ECM)-like scaffolds to be used as tracheal substitutes^{96, 148, 458, 459}. Collagen has also been widely used in respiratory tissue engineering including the use of heparin collagen cross-linked scaffolds for the regeneration of tracheal cartilage and in combination with hyaluronic acid to support the growth and maintenance of a functional and mature respiratory epithelium^{132, 138}. Although the use of natural material matrices provides a three dimensional (3D) environment rich in ECM-like components for tissue regeneration, these materials commonly lack the requisite mechanical properties needed for tracheal substitute scaffolds to become a functional implantable device to maintain patency and avoid stenosis^{206, 265, 460}. In order to overcome these limitations, the use of synthetic polymers has increased recently due to their improved mechanical as well as tunable chemical properties¹²⁹. The use of

synthetic polymers in complex clinical applications requires materials showing advanced and tunable properties that can be tailored for specific applications⁴⁶¹. For instance, several properties of these polymers can be altered in a controlled manner including mechanical strength, stiffness, molecular weight, crystallinity, degradation rate, hydrophobicity and chemical composition⁴⁶²⁻⁴⁶⁵. To date, several synthetic polymers have been used to manufacture tissue-engineered trachea (TET) constructs including polyglycolic acid (PGA), polylactic acid (PLA), poly(lactic-co-glycolic) acid (PLGA) and polytetrafluoroethylene (PTFE). However, the most extensively used synthetic polymer for tracheal regeneration is polycaprolactone (PCL) due to its high biocompatibility, biodegradability, easy printability and approval from the U.S. Food and Drug Administration (FDA) for its use in implantable devices and drug delivery systems^{144, 159, 466-471}.

Using advanced processing technologies, these natural and synthetic materials can be employed and further combined to give rise to scaffolds with specific geometries and shapes to match desired properties. Several techniques including electrospinning, casting and decellularisation have been employed to achieve 3D scaffolds suitable for tracheal implantation^{74, 186, 472-474}. Extrusion-based 3D printing has recently arisen as a popular and innovative tool for the fabrication of TE scaffolds due to its versatility, affordable price and accessibility, which allows the printing of a wide range of biocompatible materials with easy control over the printed geometry, fibre diameter and the combination of biomaterials⁴⁷⁵. Initially, 3D printing was designed for non-biological applications and therefore, the majority of the polymers first used when applying 3D printing to regenerative medicine were thermoplastic polymers, which are easy to print but usually suffer from low cellular attachment and viability^{23, 476}. Natural polymers have also been investigated as 3D printable materials including collagen, gelatin or hyaluronic acid^{477, 478}. Although these polymers present excellent biocompatibility and report high cellular attachment, they typically suffer from weak mechanical properties and low printability due to its low viscosity^{476, 479}. The use of a single material for the development of 3D printed scaffolds does not fully mimic the native ECM, resulting in the combination of synthetic and natural polymers to exploit the advantages of each materials type⁴⁷⁶. However, combination of synthetic and natural polymers can be overcome by introducing post-processing modifications into the synthetic 3D printed scaffold to increase biocompatibility and provide an enhanced

surface for cell attachment and growth⁴⁸⁰. However, most of the commonly used 3D printing materials do not allow further processing or easy surface modifications once printed. Therefore, there is a significant demand now for next generation polymers that allow post-processing modifications and functionalisation increasing material performance^{481, 482}.

This need to 3D print novel materials which allow further modification and functionalisation can be easily achieved using polyglobalide (PGL). PGL is obtained via ring-opening polymerisation from its monomer and unsaturated polyester globalide (GL)⁴⁸³. Synthesised PGL offers the advantage that it can be easily functionalised using thiol-ene chemistry in comparison to PCL or PLGA, which lack main-chain double bonds present in PGL that allow introduction of several chemical groups and surface functionalisation resulting in enhanced biofunctional materials^{172, 173, 484}. An example of this straightforward biomaterial functionalisation has already been described by de Oliveira et al. in which electrospun microfibres were cross-linked showing excellent biocompatibility properties and loaded with anti-inflammatory drugs to demonstrate its versatility¹⁷³. Although PGL scaffolds have been manufactured using electrospinning and film-based techniques, to the best of the author's knowledge PGL has never been 3D printed for tissue engineering applications^{170, 173, 485}. The potential of PGL as a versatile and biocompatible biomaterial that can support cell growth has also been demonstrated in combination with cellulose films that were used successfully in skin tissue engineering and with phosphoesters as electrospun fibres for the culture of human dermal fibroblasts^{485, 486}.

4.1.1 Aims and Objectives

The main aim of this Chapter was to evaluate the feasibility of using PGL as novel biomaterial for 3D printing of tissue engineering constructs in the first instance. Secondly, to determine its utility in respiratory tissue engineering to act as a biocompatible support in combination with the collagen and HyA bilayered (CHyA-B) scaffolds designed (Chapter 2) specifically for respiratory cell support. The specific objectives for this Chapter were:

- To evaluate whether PGL can be used as a printable material and determine the optimal parameters needed for the 3D printing process.

- To characterise PGL constructs obtained by 3D printing for its use as a novel material for tissue engineering applications.
- To determine the biocompatibility of PGL when incorporated within CHyA scaffolds and their ability to support the growth and proliferation of human mesenchymal stem cells (hMSCs).

4.2 Materials and Methods

4.2.1 Materials

Unless otherwise specified in the text, all materials and reagents were supplied by Sigma-Aldrich (Arklow, Ireland).

4.2.2 Polyglobalide (PGL) Synthesis

This work was carried out with the assistance of Dr. Ruairí Brannigan, Polymer Chemistry and Biopolymer Labs, RCSI University of Medicine and Health Sciences.

GL (Symrise, Holzminden, Germany) was used as the monomer for obtaining PGL. Four different polymers (PGL-A, PGL-B, PGL-C and PGL-D) were obtained by modifying synthesis parameters including drying time, reaction time and percentage of enzyme used to catalyse the reaction as shown in Table 4.1. For synthesising PGL, 15 g of GL and NOVOZYM 435 (*Candida Antartica* Lipase B immobilised on cross-linked polyacrylate beads) (Novozymes A/S, Bagsværd, Denmark) were weighed and dried under vacuum at 50°C. The lipase enzyme and the monomer were transferred into a two-neck round bottom flask and 30 ml of toluene AcroSeal™ 99.85% extra dry over molecular sieves (Fisher Scientific) were added. The reaction flask was purged with nitrogen and placed in an oil bath at 60°C under magnetic stirring. Dichloromethane (DCM) was added to the solution to reduce system's viscosity and was filtered using a Büchner funnel with vacuum applied to inactivate the enzyme and removed it from the polymer solution. After filtration, DCM was evaporated from the solution using a rotary evaporator. Finally, the solution containing the polymer was precipitated using 600 ml of cold methanol under magnetic stirring. The obtained polymer was dried overnight in a vacuum chamber to remove any residual methanol. After synthesis, molecular weight (M_w) and the weight average (M_n) of the produced polymer were evaluated using gel permeation chromatography (GPC). Dispersity ($\mathfrak{D}$) was then calculated as follows:

Equation 3. 1. Dispersity ($\mathfrak{D}$) calculation.

$$\mathfrak{D} = \frac{M_w}{M_n}$$

Table 4. 1. Manufacturing conditions used to synthesize PGL-A, PGL-B, PGL-C and PGL-D.

Polymer	Drying	Reaction Time	% Enzyme (<i>in relation to monomer</i>)
PGL-A	7 days	7 days	0.1% wt
PGL-B	3 days	7 days	0.1% wt
PGL-C	24 h	4 h	0.1% wt
PGL-D	24 h	4 h	6% wt

4.2.3 3D Printing of Polyglobalide (PGL)

Polymer constructs were 3D printed using a Biobot1 printer (Allevi, Philadelphia, PA). Metal syringes were rinsed twice in chloroform, 99+% Acros Organics™ (Fisher Scientific) and 70% v/v ethanol prior to use to remove any residual polymer. Synthesised PGL was heated to 70°C and extruded using a 25G metal needle (Inner diameter 0.26 mm, Micron-S Fisnar; ECT Adhesive & Dispensing Solutions, Dublin, Ireland). Python was used to generate the g-codes needed for printing the geometries of the 3D constructs. Two geometries were 3D printed: a lattice-like square mesh and a disc geometry (Figure 4.1). The lattice geometry consisted of 8 layers with a total height of 2 mm and a width of 7.5 mm. The disc geometry consisted of 18 layers with a total height of 4.5 mm and a diameter of 10 mm. PCL, with a M_w of 37 kDa (VWR, Ballycoolin, Ireland) and 50 kDa (CAPA™ 6500; Perstorp, Malmö, Sweden), were used as control for all experiments. PCL was chosen as the control for printing experiments due to its widespread use in tissue engineering applications as well as its good mechanical properties, biocompatibility and approval by the FDA^{179, 487}. The lattice geometry was employed for assessing PGL printability and post-printing characterisation, while the disc geometry was used to evaluate the polymer's biocompatibility. PCL 37 kDa and 50 kDa were 3D printed at 100°C, using 70 PSI and 80 PSI and a printing speed of 280 and 80 mm/min respectively.

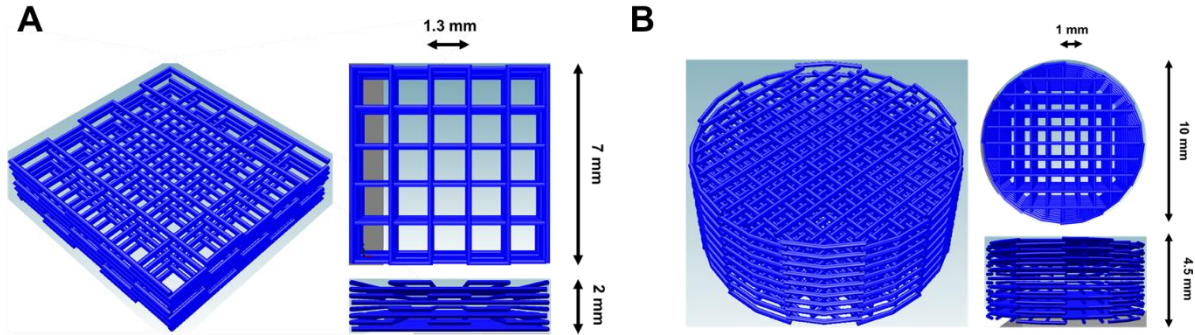


Figure 4. 1. Schematic designs used for 3D printing PGL constructs. (A) Lattice geometry used for optimizing PGL 3D printing parameters and post-printing characterisation. (B) Disc geometry used for reinforcing CHyA scaffolds to evaluate PGL biocompatibility.

4.2.4 Post Printing Characterisation of Polyglobalide (PGL)

The lattice-like geometry was used to characterise the printability of PGL. Weight uniformity of printed constructs, their mechanical properties and thermal properties were evaluated after 3D printing.

4.2.4.1 Construct Weight Evaluation

The weight of printed constructs from different batches was determined to evaluate the ability of PGL to be 3D printed in a uniform manner and to demonstrate a consistent material deposition throughout the printing process. 3D printed lattice constructs were detached from the printing platform using a scalpel and were weighed using a PB153-S balance (Mettler Toledo, Worthington, US).

4.2.4.2 Mechanical Characterisation

Lattice 3D printed constructs were mechanically characterised with a mechanical testing machine Z050 (Zwick-Roell, Haan, Germany). To determine the mechanical strength of the designs using different polymers, the 3D printed constructs were tested using uniaxial compression for 3 cycles, a 50 N load cell and 20% strain. The compressive modulus was then calculated using the stress-strain curves. Briefly, the modulus (E) in N was calculated as the slope of the stress-strain curve. Compressive Young's modulus was defined as the applied force (N) per surface area of each sample (m^2) and strain was calculated as percentage of displacement of the sample during compression.

4.2.4.3 Differential Scanning Calorimetry

This work was carried out with the assistance of Dr. Ruairí Brannigan, Polymer Chemistry and Biopolymer Labs, RCSI University of Medicine and Health Sciences.

The thermal properties of the 3D printed constructs were characterised using differential scanning calorimetry (DSC) and a Q200 Differential Scanning Calorimeter (TA Instruments, Asse, Belgium). DSC heating and cooling cycles were run in triplicate in series between -40 and 150°C under a nitrogen atmosphere at a heating rate of $\pm 10^{\circ}\text{C}/\text{min}$ in a $40\ \mu\text{l}$ aluminum crucible. The melting temperatures were determined using the second heating cycle in order to negate thermal history imparted on materials.

4.2.4.4 Fibre Diameter and Quantification

To evaluate the mean fibre diameter of the lattice geometry for each polymer, brightfield images were captured using a DFC420 C microscope (Leica, Wetzlar, Germany). Five constructs of each polymer were used and three images were captured for each one using a 4X magnification. The brightfield images were analysed to estimate the fibre mean diameter using FIJI⁴⁸⁸.

4.2.5 NaOH and EDAC Treatment Effect on Polymer-CHyA Disc Scaffolds

Current protocols for polymer-reinforced scaffolds used in RCSI University of Medicine and Health Sciences TERG utilise sodium hydroxide (NaOH) treatment to improve collagen infiltration into the polymer 3D printed network. Treatment of PCL with NaOH has been demonstrated to improve the polymer's hydrophilicity and surface roughness to increase collagen infiltration⁴⁸⁹. To evaluate the effect of NaOH treatment of PGL constructs before freeze-drying, 3D printed constructs were submerged in 3 M NaOH for 4, 12, 24 and 48 h to determine the effect on the mechanical properties and integrity of the constructs. CHyA scaffolds are cross-linked using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and N-Hydroxysuccinimide (NHS) as described in Chapters 2 and 3¹³². To evaluate the effect of EDAC cross-linking on PGL constructs, 3D printed constructs were treated for 2 h with 6 mM EDAC and N-Hydroxysuccinimide (NHS) in a 5:2 molar ratio of EDAC:NHS. Treated and non-treated constructs were characterised using compression mechanical testing as described in Section 4.2.4.2

4.2.6 Freeze-drying of Polymer-CHyA Disc Scaffolds

Due to the high hydrophobicity of PGL and to evaluate the feasibility of reinforcing collagen-based scaffolds with polymers to increase its mechanical strength, 3D printed PGL discs were freeze-dried in combination with collagen and hyaluronic acid (CHyA) scaffolds⁴⁹⁰. CHyA slurry was obtained as outlined in Chapter 2 by blending a suspension of 0.5% w/v type I collagen with 0.004% w/v HyA sodium salt from *Streptococcus equi* in 0.5 M acetic acid (AcOH) as previously described¹³². The CHyA slurry was degassed under a vacuum to remove air bubbles created during the homogenisation process. 300 µl of CHyA slurry were pipetted into customised metal moulds with a diameter of 11 mm and 3D printed discs of PGL-D, PCL 37 kDa and PCL 50 kDa were placed on top of the slurry and allow to sink to the bottom of the mould to manufacture PGL-D-CHyA, PCL-37-CHyA and PCL-50-CHyA disc scaffolds. CHyA non-reinforced discs were obtained by adding 400 µl of slurry and were used as controls to evaluate the effect of polymer reinforcing. Prior to combination with CHyA-B slurry, the 3D printed constructs were treated with 3 M NaOH in distilled H₂O.

4.2.7 Pore Size Analysis of Polymer-CHyA Disc Scaffolds

The porosity of PGL-D-CHyA, PCL-37-CHyA and PCL-50-CHyA disc scaffolds and the effect of reinforcing CHyA scaffolds with the different polymers was evaluated by calculating the scaffold's mean pore size, which is crucial for cellular adhesion and growth¹⁷⁸. To estimate the mean pore diameter, scaffolds from different 3D printing and freeze-drying batches were analysed with a previously described technique^{491, 492}. Samples were embedded using a JB[®]-4 Embedding Kit (Polysciences, Hirschberg an der Bergstrasse, Germany). Scaffolds were hydrated overnight in 2 ml of Dulbecco's phosphate-buffered saline (DPBS) and fixed using 10% v/v neutral buffered formalin solution. Briefly, scaffolds were incubated with 2 ml DPBS:formalin 1:1 for 1 h at RT and incubated overnight at 4°C. Scaffolds were then dehydrated with distilled H₂O for 30 min twice, 50% v/v ethanol for 30 min, 70% v/v ethanol for 1 h 30 min, 95% v/v ethanol for 1 h 30 min and 100% v/v ethanol for 2 h 15 min. Following rehydration, samples were equilibrated for 12 h at 4°C using equilibration solution and were incubated with infiltration solution for 8 h at 4°C three times. Infiltration solution was obtained by dissolving benzoyl peroxide in JB-4 Embedding Solution A to a final concentration of 12.5 mg/ml. Equilibration solution was a 1:1 dilution of infiltration

solution and 100% v/v ethanol. After the infiltration steps, the scaffolds were embedded in JB-4 moulds using 2 ml of embedding solution which consisted of a 1:25 dilution of JB-4 Embedding Solution B in infiltration solution. Moulds with samples were stored at 4°C until further processing. Sections of 10 µm width from embedded scaffolds were obtained using a RM2255 automated microtome (Leica, Dublin, Ireland). Ten sections per scaffold were obtained comprising a total depth of 1000 µm of the scaffold. Sections were then stained using 0.5% v/v toluidine blue solution in distilled H₂O. Images were obtained using a Nikon Eclipse 90i microscope (Nikon, Tokyo, Japan) and a magnification of 10x.

4.2.8 Scaffold Ultrastructure Characterisation

To evaluate collagen infiltration and incorporation into the 3D printed disc, polymer reinforced scaffolds were examined using scanning electron microscopy (SEM) at the Advanced Microscopy Laboratory (Trinity College Dublin, Dublin, Ireland). Imaging of the scaffolds was performed using a Tescan Mira XMU scanning electron microscope (Tescan, Brno-Kohoutovice, Czechia). Images were captured at 5 kV using secondary electron mode, taken at a working distance between 12-18mm.

4.2.9 Biocompatibility Assessment of PGL-D-CHyA Disc Scaffolds

Prior to cell seeding and evaluation of biocompatibility, the scaffolds were sterilised in an ethylene oxide Anprolene AN74 Series gas steriliser (Andersen Sterilizers, Haw River, NC) and chemically cross-linked as described in Section 4.2.5.

4.2.9.1 Cell Selection and Culture Media

The ability of PGL-D-CHyA disc scaffolds to support the growth of hMSCs was evaluated. hMSCs were isolated and cultured from iliac crest bone marrow aspirated obtained from Lonza (Basel, Switzerland) as previously described^{493, 494} or purchased from Promocell (Heidelberg, Germany). hMSCs were cultured in 175 cm² CELLSTAR® cell culture flask following revival, seeded at a density of 1 x 10⁶ cells per T175 flask and passaged when they reach 80% confluency using 0.25% Trypsin-EDTA solution. hMSCs were cultured in low glucose Dulbecco's Modified Eagle's Medium supplemented with 10% v/v foetal bovine serum (FBS), 100 U/ml penicillin/streptomycin and 1% v/v Primocin™ (InvivoGen, San Diego, USA). Unless otherwise stated, all cell culture and incubation steps were performed at 37°C and 5%

CO₂ in a humidified atmosphere. Polymer-reinforced and CHyA disc scaffolds were seeded with 3×10^5 cells/scaffold, seeding 1.5×10^5 cells per side and allowing for 2 h incubation in between sides in a suspension 24 well TC Plate (Sarstedt, Nümbrecht, Germany). Scaffolds were then transferred to a new plate and medium was exchange twice a week with 2 ml of fresh medium. At day 1, 7 and 14 samples and/or scaffolds were taken for evaluating the cell metabolic activity, lactate dehydrogenase (LDH) release, DNA quantification as well as for cell imaging.

4.2.9.2 Cell Metabolic Activity

Metabolic activity from cells grown on scaffolds was evaluated at day 1, 7 and 14 of culture using alamarBlue™ Cell Viability Reagent (Biosciences, Dublin, Ireland). Briefly, 2 ml of 1X alamarBlue™ solution was added to each scaffold and incubated for 2 h. Following incubation time, 100 µl of supernatant were transferred to a Corning® 96-well black flat bottom polystyrene not treated microplate and fluorescence was measured at 545 nm excitation and 590 nm emission wavelengths in a Tecan Infinite M Plex plate reader (Tecan, Männedorf, Switzerland).

4.2.9.3 Lactate Dehydrogenase (LDH) Release

Cell culture supernatant was collected and used to evaluate PGL's potential toxic effect on hMSCs using Invitrogen™ LDH Cytotoxicity Assay (Fisher Scientific). 50 µl of supernatant were pipetted into a Corning® 96-well clear flat bottom polystyrene not treated microplate and incubated with 50 µl of Reaction Mixture, which was a combination of 600 µl of Assay Buffer Stock Solution with 11.4 ml of Substrate Stock Solution. Samples were incubated for 30 min at room temperature before adding 50 µl of Stop Solution. Absorbance was measured at 490 nm and 680 nm in a Tecan Infinite M Plex plate reader. LDH activity was obtained by subtracting the 680 nm absorbance value from the 490 nm value and normalised to the LDH activity from untreated cultures for each culture condition.

4.2.9.4 DNA quantification

Seeded 3D printed discs were processed at day 1, 7 and 14 using Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, Paisley, UK) to quantify double-stranded DNA (dsDNA) from the scaffolds. Scaffolds were rinsed in DPBS and submerged in 1 ml of lysis buffer, which consisted of 1% v/v Triton X-100 0.2 M Na₂CO₃ in distilled H₂O and

freeze thaw three times at -80°C. 100 µl of sample was added to a Corning® 96-well black flat bottom polystyrene not treated microplate and incubated with 100 µl of PicoGreen working solution; 1:200 dilution of PicoGreen Stock Solution in 1X TE solution in distilled H₂O. Fluorescence was read by exciting at 485 nm and reading at 538 nm. dsDNA concentration was estimated using a standard calibration curve following manufacturer's instructions.

4.2.9.5 Live/Dead Imaging

At day 14 of culture, a modified version of Live/Dead cell viability staining was used to evaluate the biocompatibility of PGL-D-CHyA scaffolds. Scaffolds were washed twice in 2 ml of DPBS and incubated for 20 min protected from light with 2 ml of 4 µM ethidium homodimer-1 from the LIVE/DEAD® Viability/Cytotoxicity Kit (BioSciences), which acted as the dead compound. Scaffolds were then washed with DPBS, fixed with 2 ml of 10% v/v neutral buffered formalin solution for 10 min and incubated with 1:1000 DAPI readymade solution in DPBS for 10 min protected from light. Scaffolds were then stored at 4°C until imaging using an Axio Examiner.Z1 confocal microscope (Carl Zeiss, Cambridge, UK). Images were processed and examined using FIJI ⁴⁸⁸.

4.2.10 Statistical Analysis

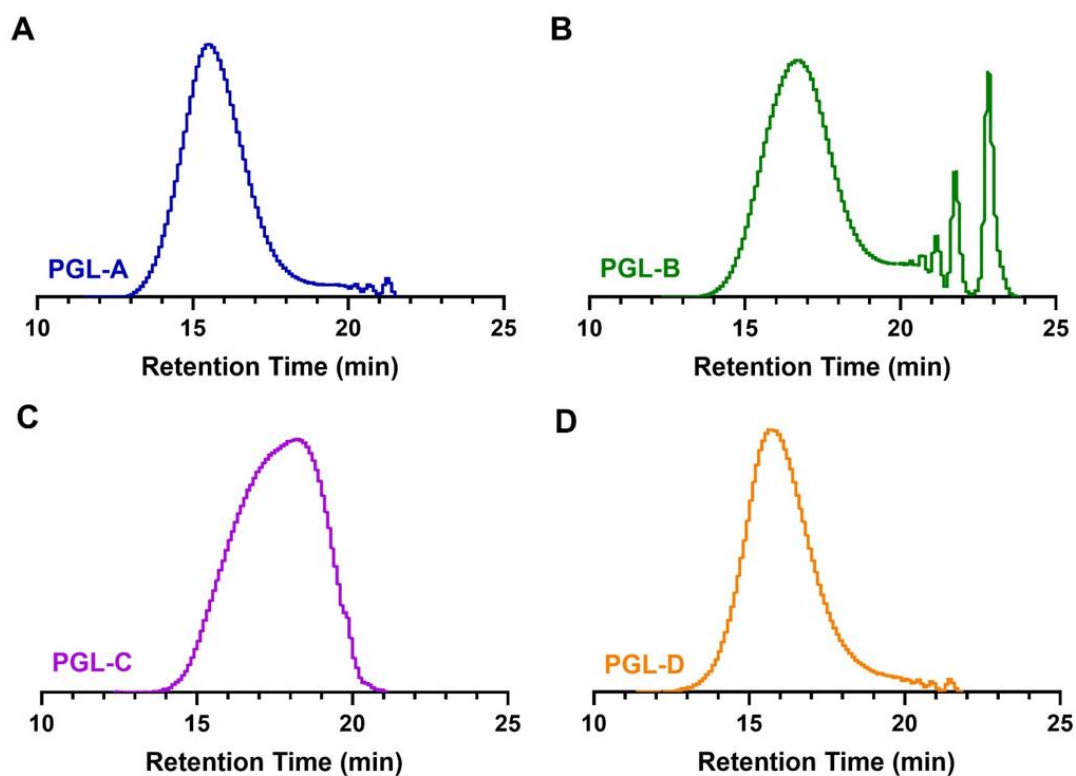
Quantitative data obtained were analysed using Microsoft Excel and GraphPad Prism 7.0 Software. In cases of analysis between two groups, statistical difference was assessed by two-tailed Student t-test. For multiple groups, statistical difference between groups was assessed by 2-way Analysis of Variance (ANOVA).

4.3 **Results**

4.3.1 **PGL Synthesis**

This work was carried out by Dr. Ruairí Brannigan, Polymer Chemistry and Biopolymer Labs, RCSI University of Medicine and Health Sciences.

PGL was synthesised from its monomer GL by modifying synthesis parameters including drying time, reaction time and percentage of enzyme used to catalyse the reaction. These synthesis modifications resulted in polymers of PGL showing different M_w and dispersity measured using GPC (Figure 4.2). PGL-A and PGL-D had similar M_w of 66 kDa and an acceptable dispersity of 3.695 and 3.23, respectively. On the other hand, PGL-B and PGL-C had a lower M_w of 29.06 and 20.06 kDa, respectively. PGL-B presented a higher dispersity of 18.15 in comparison to PGL-C (Figure 4.2E). The uniformity of polymer M_w distribution was further analysed using GPC as shown in (Figure 4.2A-D). The presence of a homogenous polymer distribution was observed for PGL-A, PGL-C and PGL-D, while a broad range of polymer M_w was seen for PGL-B in the GPC distributions.



E

Polymer	M_n	M_w	Dispersity (ϑ)
<i>PGL-A</i>	18,000 g/mol	66,520 g/mol (66.52 KDa)	3.695
<i>PGL-B</i>	1,601 g/mol	29,060 g/mol (29.06 KDa)	18.15
<i>PGL-C</i>	8,426 g/mol	20,060 g/mol (20.06 KDa)	2.445
<i>PGL-D</i>	20,650 g/mol	66,700 g/mol (66.70 KDa)	3.23

Figure 4. 2. Gel permeation chromatography (GPC) from synthesised PGL: (A) PGL-A, (B) PGL-B, (C) PGL-C and (D) PGL-D. (E) M_n , M_w and dispersity of synthesised PGL-A, PGL-B, PGL-C and PGL-D.

4.3.2 3D Printing of Polyglobalide (PGL) polymers

Once the different PGL polymers were manufactured, their printability was assessed and 3D printing conditions optimised as shown in Figure 4.3A. To determine their printability, the lattice geometry was used in the first instance. This geometry consisted of 8 layers with a total height of 2 mm and a width of 7.5 mm. The printing speed and pressure of the 3D printer was optimised using 70°C as the standard temperature, above the previously reported melting point for PGL (45-49°C) in order to improve polymer flow through the extruding tip^{495, 496}. Pressure needed for extruding PGL-C was set to 5 PSI to ensure precision while using 500 mm/min printing speed. However, it was found that PGL-C was not suitable for 3D printing as the formation of a defined squared mesh was not achieved. The extruded polymer did not solidify completely after melting and printing, therefore it was removed for the following experiments. PGL-A and PGL-B were extruded using a pressure of 15 PSI and a printing speed of 300 and 180 mm/min respectively. Finally, PGL-D was printed using a higher pressure in comparison to PGL-A and PGL-B of 55 PSI and a printing speed of 350 mm/min to achieve defined squared mesh geometry without the presence of fused fibres. As shown in Figure 4.3B, PGL-D was the polymer that enabled the most precise print in comparison to the commonly used PCL controls employed in 3D printing applications.

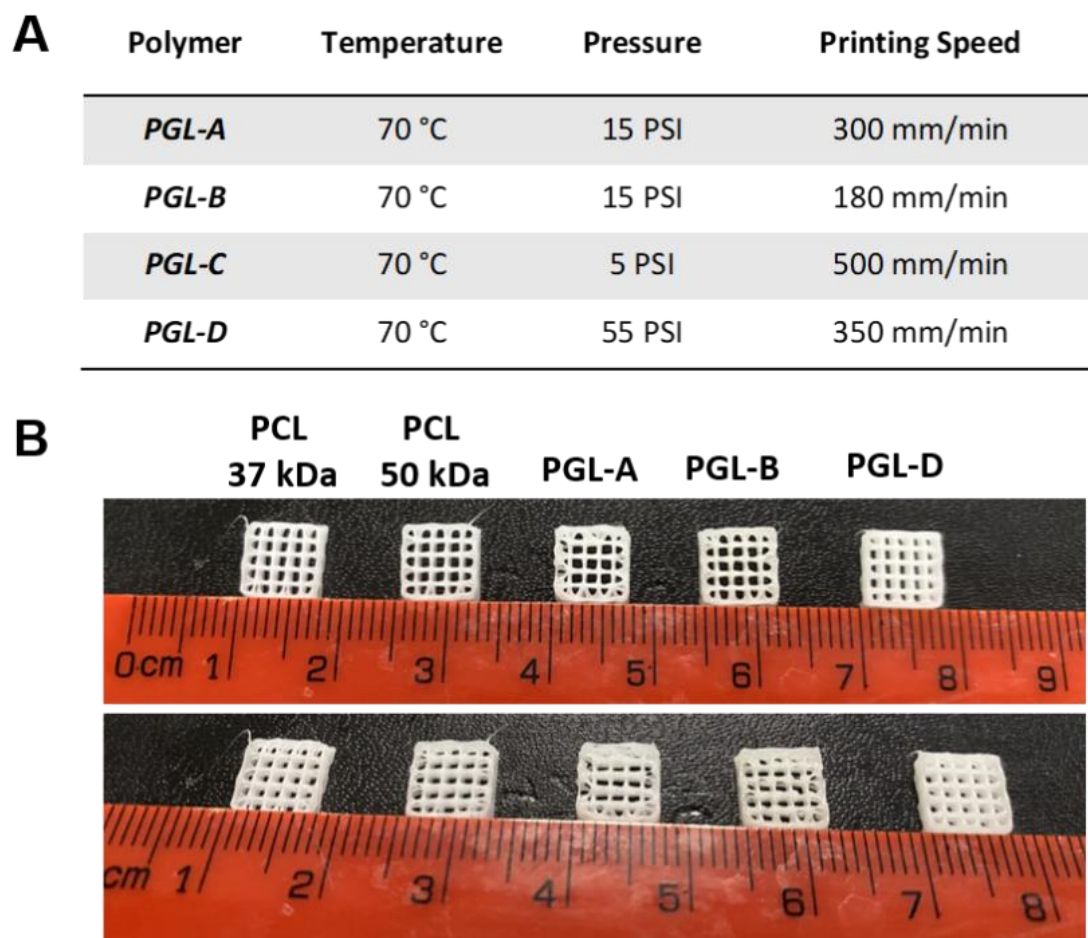


Figure 4. 3. 3D printing optimisation of PGL. (A) Printing conditions used to 3D print PGL-A, PGL-B, PGL-C and PGL-D. (B) Macrographs of PCL and PGL printed constructs.

4.3.3 Post-printing Characterisation of Polyglobalide (PGL) Lattice Constructs

Weighting of the 3D printed constructs was used to determine the reproducibility of the printing process. PGL-A and PGL-D polymers could be reproducibly 3D printed and the resulting constructs easily controlled to produce uniform constructs over different prints (Figure 4.4A). PGL-A and PGL-D constructs had a similar weight range in comparison to PCL 37 kDa, with a narrow range distribution of weights. However, this was not observed for PGL-B constructs, which had a wide range of weights from 40 to 70 mg/construct, which was significantly different and higher to PCL 37 kDa. This highlighted a differential material deposition and therefore different amount of extruded material between different print runs, albeit using the same printing conditions. This could be explained by the wide range of M_w observed for PGL-B after GPC (Figure 4.4A, Figure 4.2B). The Young's modulus from the 3D printed lattice constructs was determined to evaluate the mechanical properties of each of the PGL polymer constructs after the printing process (Figure 4.4B). All PGL 3D printed constructs had significantly reduced mechanical properties in comparison to PCL 37kDa, especially PGL-D. However, when compared to PCL 50kDa with a similar M_w to PGL-A and PGL-D, no significant differences were observed in the Young's modulus.

A thermal analysis was carried out using the 3D printed constructs to better evaluate PGL properties comparing it to PCL and to determine if the printing process had an effect on the thermal properties of the polymer. As can be seen from the DSC thermogram, the PGL constructs (A-D) have a narrow melting temperature (T_m) range between 45-47°C, and crystallisation temperature (T_c) range between 29-31°C (Figure 4.4C). Notably, the T_m of PGL is considerably lower than that of PCL (57°C), which can be attributed to the unsaturated nature of PGL¹⁷¹. Easily rotating bonds (i.e. single bonds adjacent to cis-C=C bonds) confer greater chain flexibility and therefore lead to lower melting points. In addition, PCL exhibited a lower melting exotherm than the PGL samples which is indicative of a lower crystallinity. Interestingly, the melting exotherm for PGL-B appeared to be larger than that of PGL-A and PGL-C, indicative of an increase in crystallinity.

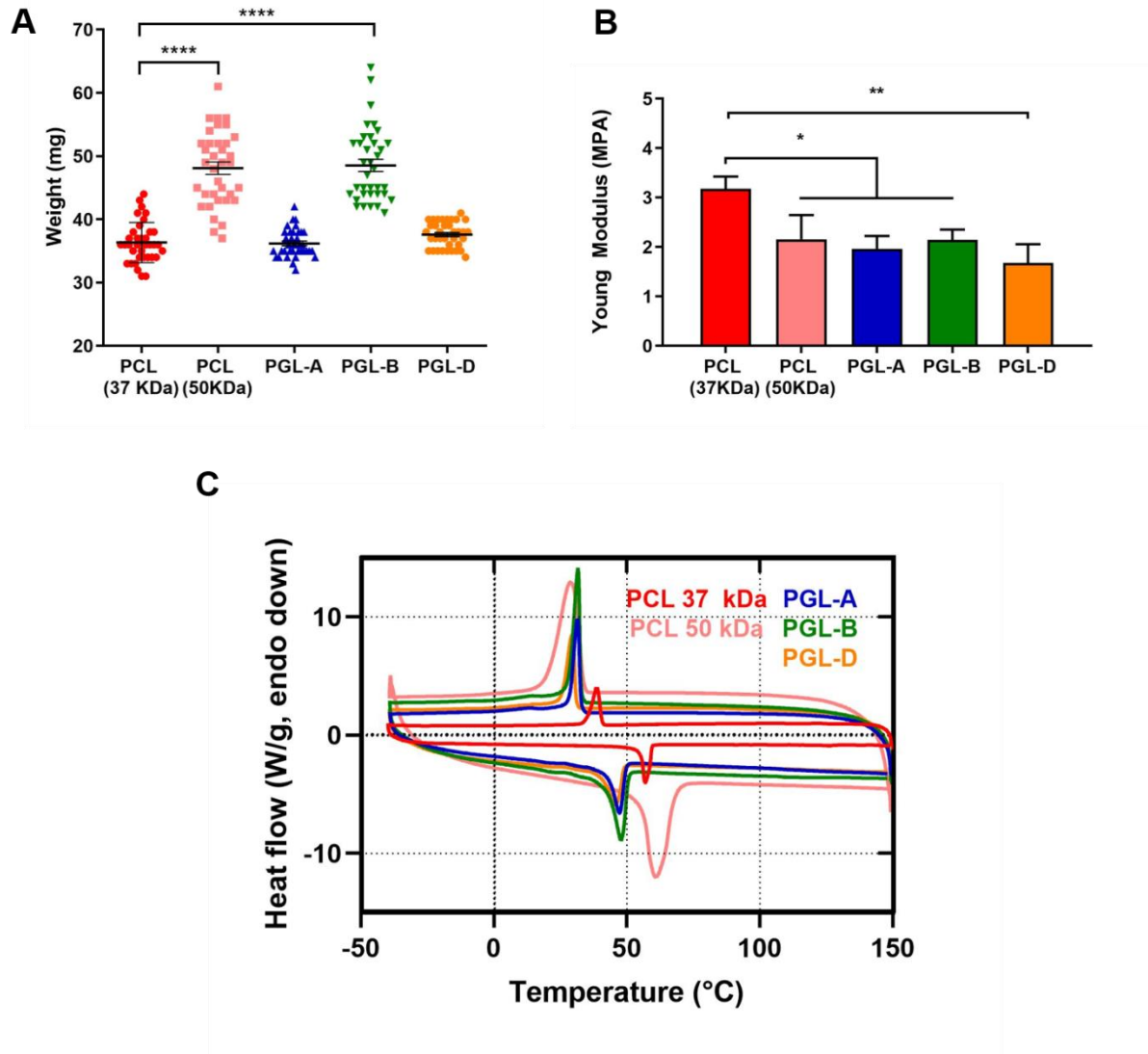


Figure 4. 4. Post printing characterisation of 3D printed PGL lattice constructs in comparison to PCL 37 kDa and 50 kDa. (A) Weight uniformity of the printed constructs (n=30). (B) Mechanical compression of 3D printed constructs using 20% Strain and 50N load cell. Results displayed as mean $\pm$ SEM (n = 6). (C) Differential scanning calorimetry. (*p<0.05, **p<0.01, ****p<0.0001).

The fibre mean diameter of the constructs was evaluated as a final marker for the printability of PGL (Figure 4.5). PCL constructs showed a fibre diameter of 309.3 ± 4.6 and $382.5 \pm 15.77 \mu\text{m}$ for the 37 and 50 kDa polymer respectively. On the other hand, fibre diameters equal to 351.6 ± 3.6 , 340.3 ± 13.12 and $365.3 \pm 4.2 \mu\text{m}$ were measured for PGL-A, PGL-B and PGL-D constructs (Figure 4.5A). Due to the consistent and enhanced printing properties (weight uniformity, printing speed and printing reproducibility) of PGL-D in comparison to PGL-A, PGL-B and PGL-C, it was decided that it would be the polymer of choice brought forward for further experiments in this Chapter focusing on reinforcement of CHyA scaffolds for biocompatibility.

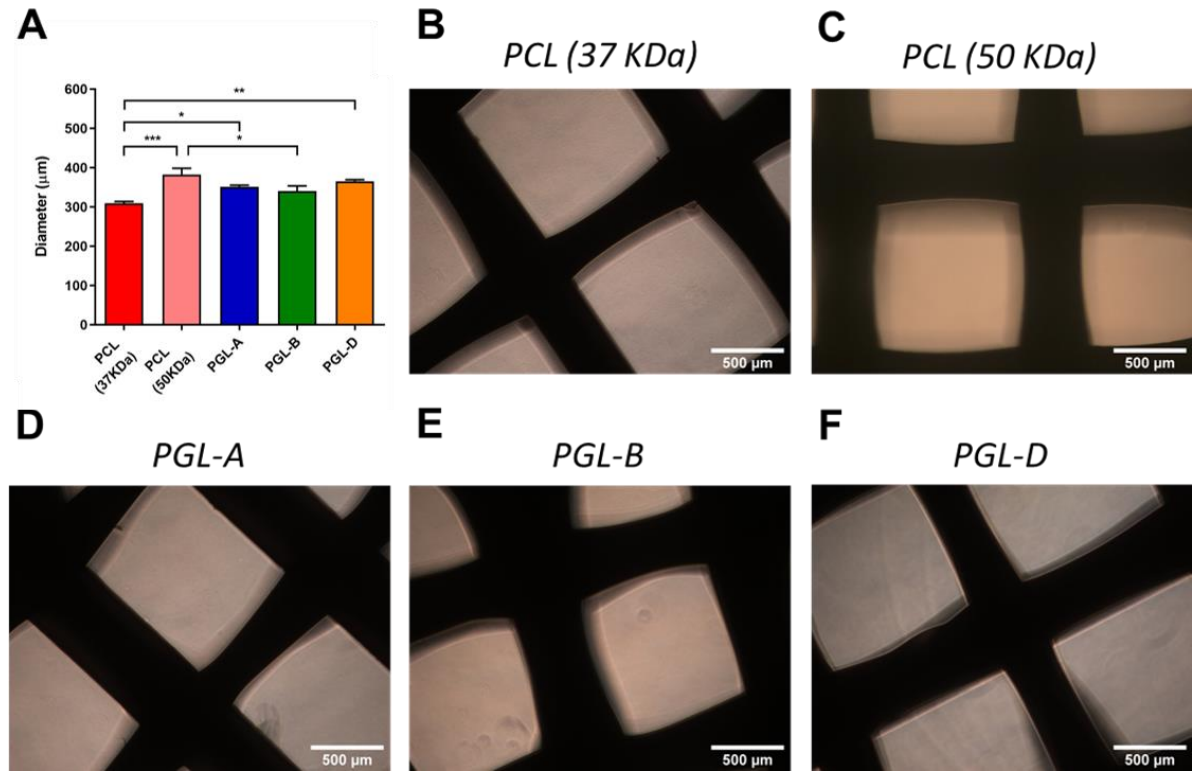


Figure 4. 5. Post printing characterisation of 3D printed PGL lattice constructs in comparison to PCL 37 kDa and 50 kDa. (A) Mean fibre diameter of printed constructs. (B-F) Representative brightfield visualisation of printed constructs. Results displayed as mean $\pm$ SEM ($n = 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.4 Incorporation of 3D Printed PGL into CHyA Scaffolds

CHyA scaffolds were incorporated into the 3D printed geometry of PGL in order to take advantage of the properties of the natural polymer for cell growth and attachment. EDAC cross-linking is used in collagen-based scaffolds to increase compressive modulus with the aim of increase cell attachment and proliferation as previously reported³³⁰. To ensure cross-linking with EDAC did not have any detrimental effect on the 3D printed construct used to reinforce the CHyA scaffold, PGL discs were chemically cross-linked with EDAC for 2 h and the changes in Young's Modulus evaluated after cross-linking (Figure 4.6A). Chemical cross-linking with EDAC:NHS did not alter the mechanical properties of PGL 3D printed constructs. This was also seen in the PCL 37 kDa and 50 kDa controls, with changes that were not statistically significant (p value = 0.7031 and 0.8751 respectively), highlighting the possibility of chemically cross-linking the CHyA network formed around the 3D printed structure after freeze-drying.

In order to increase the infiltration of collagen within the 3D printed structure, current protocols used in RCSI TERG employ a pre-treatment of PCL constructs using 3 M NaOH for 48 h to make the surface of the construct more hydrophilic and therefore increase infiltration of collagen. Albeit treatment of PCL with NaOH has been shown to increase cellular attachment without substantial changes in mechanical properties⁴⁹⁷, the effect of NaOH treatment in PGL disc constructs remains unknown and therefore was evaluated for 4, 12, 24 and 48 h (Figure 4.6B). A decrease in the Young's Modulus was observed after 12 h treatment with a significant decrease after treatment for 24 h and 48 h. Handling of 3D printed constructs post-NaOH treatment showed delamination and collapse of the construct highlighting that the decrease of mechanical properties arose from delamination of the structure rather than degradation of the polymer network. As this was also seen for constructs treated with NaOH for 12 h, 4 h treatment with 3 M NaOH was selected before freeze-drying to increase collagen infiltration within the 3D printed polymer network.

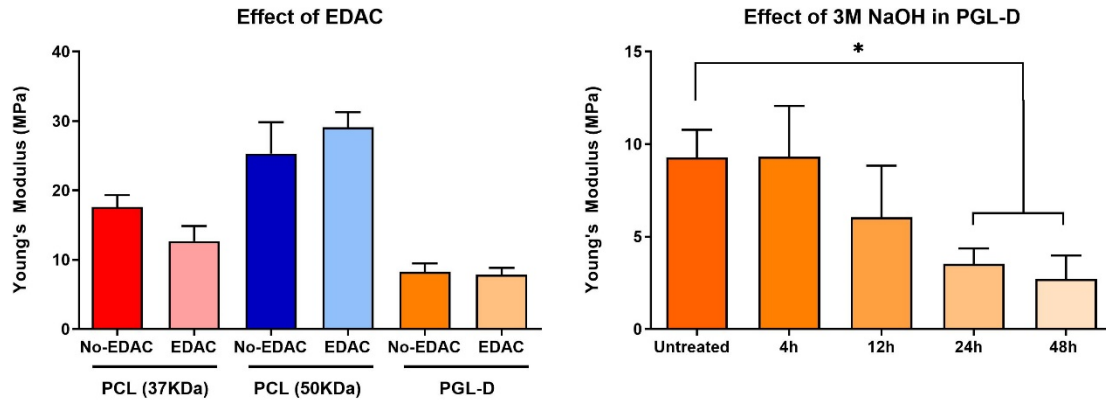


Figure 4. 6. Effects of EDAC and NaOH treatment on the mechanical properties of PGL-D printed discs using 20% strain and 50N load cell. (A) Mechanical properties before and after EDAC treatment of the 3D printed disc scaffolds (n=5). (B) Mechanical properties of PGL 3D printed constructs treated with 3M NaOH for 4, 12, 24 and 48 h (n=3, **p<0.01).

3D printed disc geometries were submerged in CHyA slurry and the combination was freeze-dried to achieve infiltration of the slurry within the printed disc. The combination and incorporation of the CHyA slurry with the 3D printed disc constructs was evaluated using SEM imaging and the pore size of the freeze-dried scaffolds was evaluated (Figure 4.7, Figure 4.8). As shown in Figure 4.7, the CHyA solution was effectively freeze-dried around the 3D printed PCL and PGL constructs. This collagen distribution and the characteristic porous network around the printed constructs was further confirmed via toluidine staining (Figure 4.8A-D). Furthermore, analysis of the mean pore size of PGL-D-CHyA, PCL-37-CHyA and PCL-50-CHyA disc scaffolds revealed no significant differences upon incorporation of the 3D printed geometry when compared to non-reinforced CHyA scaffolds (Figure 4.8E).

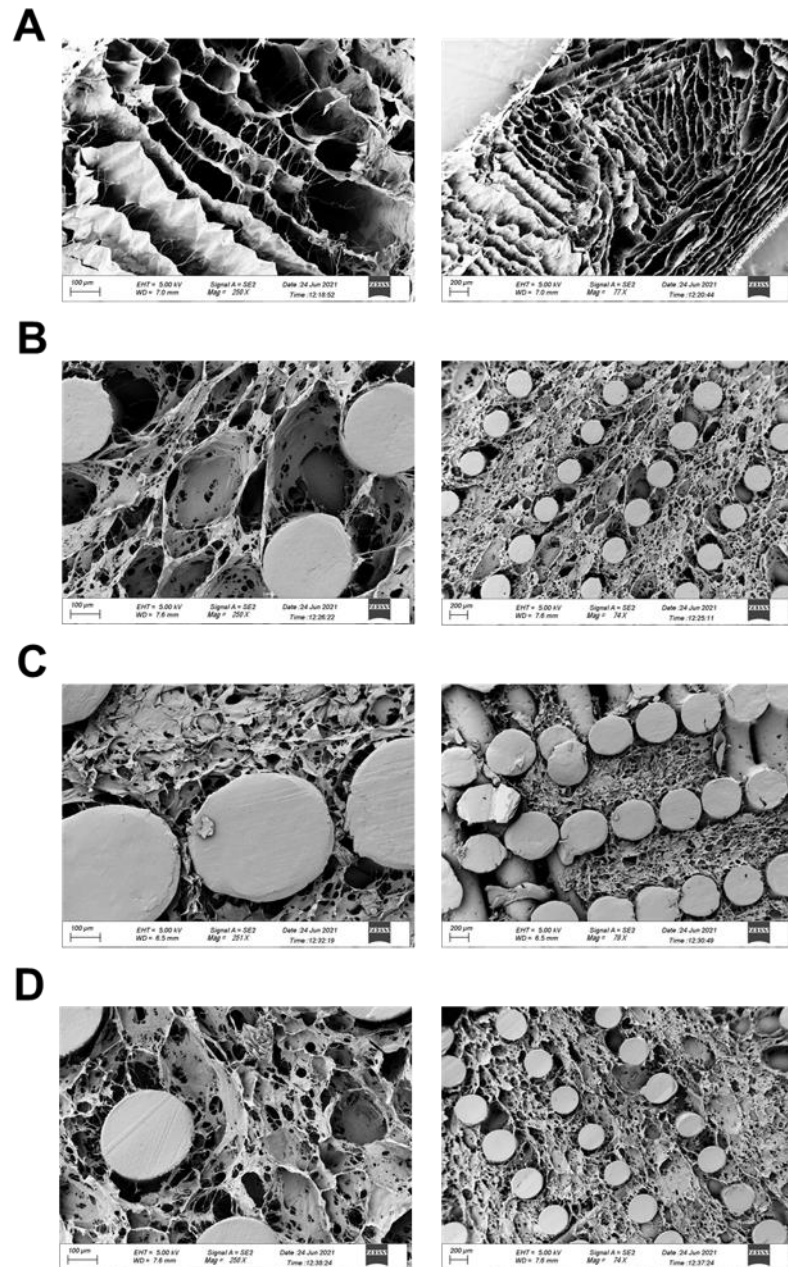


Figure 4. 7. SEM imaging of (A) CHyA, (B) PCL-37-CHyA, (C) PCL-50-CHyA and (D) PGL-D-CHyA disc scaffolds using a 250X and 77X magnification.

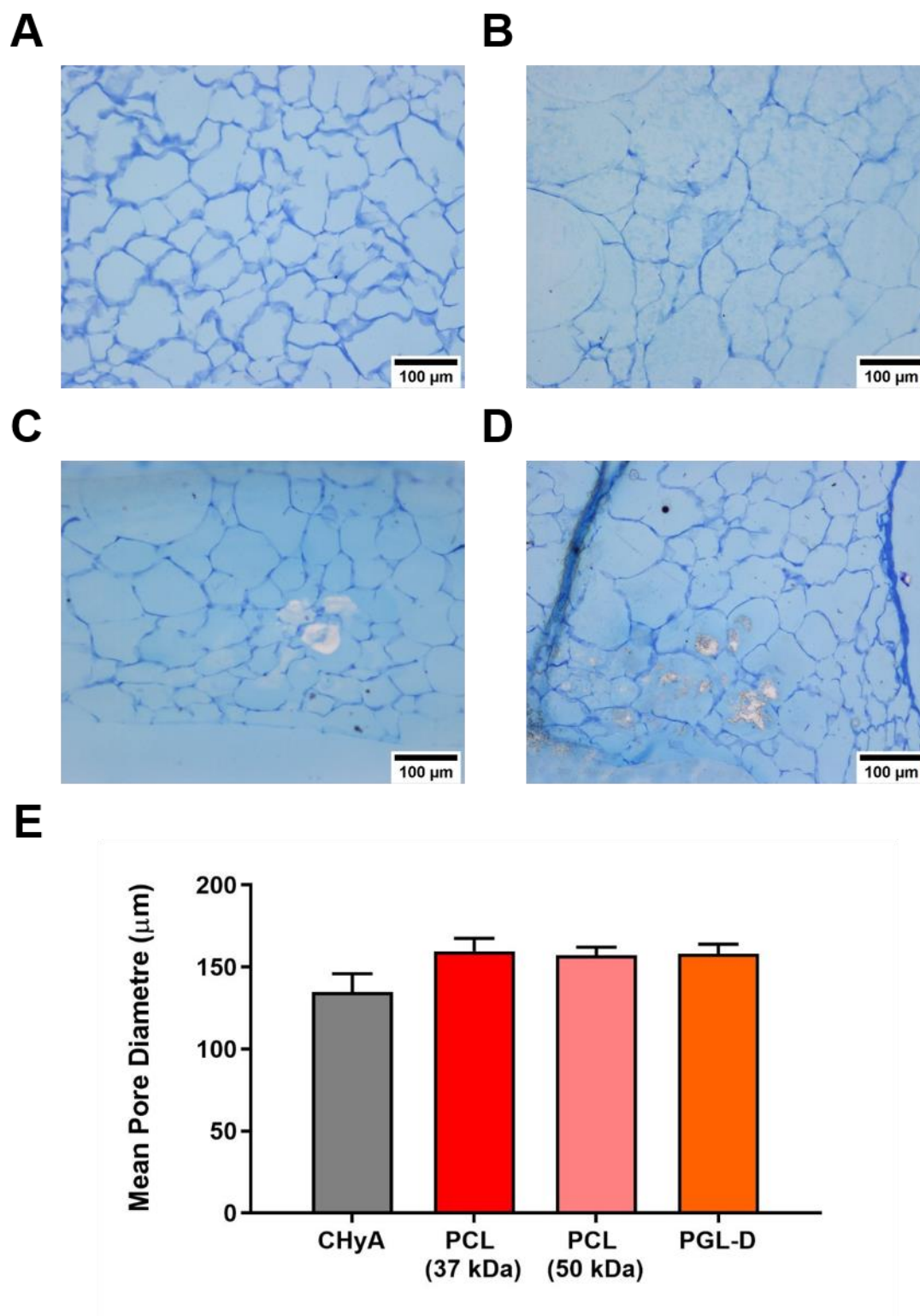


Figure 4. 8. Pore size analysis of CHyA reinforced 3D printed discs: (A) CHyA, (B) PCL-37-CHyA, (C) PCL-50-CHyA (D) PGL-D-CHyA disc scaffolds. (E) Mean pore diameter of freeze-dried scaffold discs. Results displayed as mean $\pm$ SEM (n = 5).

4.3.5 PGL-CHyA Scaffold Biocompatibility

Metabolic activity from CHyA disc scaffolds reinforced with 3D printed constructs was measured in the first instance to determine the effect of the polymer construct support used on the ability of CHyA to support cell attachment. This was further examined to compare the effect of the different polymers used between them (Figure 4.9A). In order to better determine the cell proliferation over time for each scaffold, the values measured at day 7 and 14 were normalised to those obtained at day 1 for each culture condition (Figure 4.9B). Non-reinforced CHyA disc scaffolds showed high metabolic activity readings at day 1, which was further increased by day 7 and 14. PCL-37-CHyA and PCL-50-CHyA disc scaffolds showed a similar profile for cell growth over the culture period, which was comparable to CHyA scaffolds. For each, the metabolic activity measured at day 7 and 14 was significantly increased in comparison to day 1, showing the ability of the PCL-37-CHyA and PCL-50-CHyA disc scaffolds to support hMSC growth over time. PGL-D-CHyA disc scaffolds showed significantly lower cell metabolic activity at day 1, 7 and 14 in comparison to CHyA alone and PCL-CHyA disc scaffolds. After normalizing the values to the ones obtained at day 1, a trend for increased metabolic activity at day 7 was observed albeit not significant for PGL-D-CHyA disc scaffolds (Figure 4.9B). The cell metabolic activity on PGL-D-CHyA disc scaffolds did not show a significant decrease over time, which would have demonstrated a toxic effect of the PGL network on the seeded cells. These results demonstrated that the presence of PGL within the CHyA disc scaffold appeared to compromise initial cell attachment in comparison to the PCL. Once attached to PGL, the cells appeared to be metabolically active at day 7 and 14. Together with the absence of a reduced cell activity after normalisation to day 1, it demonstrated the biocompatibility of PGL to support the growth of cells that were able to attach to the scaffold showing no detrimental toxic effect.

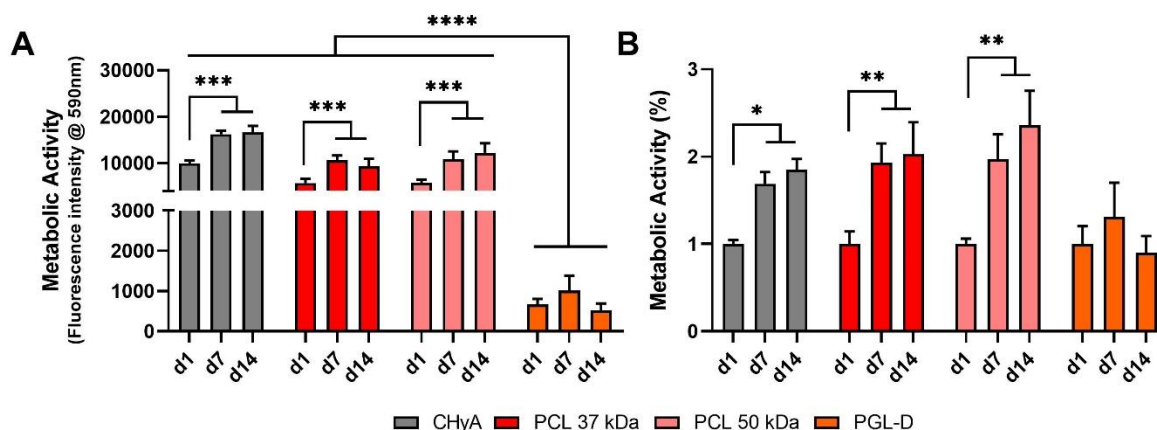


Figure 4.9. Cell metabolic activity measured from CHyA, PCL-37-CHyA, PCL-50-CHyA and PGL-D-CHyA disc scaffolds seeded with hMSCs. (A) Cell metabolic activity values measured at day 1, 7 and 14. (B) Normalised values to each the corresponding day 1 of each CHyA and polymer-reinforced CHyA scaffolds (n=3, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Contrary to cell metabolic activity evaluated using alamarBlue™ assay, LDH release was measured to detect any toxic effect after reinforcing CHyA scaffolds as LDH is a good indicator of cell damage and death⁴⁹⁸. LDH release from PCL-CHyA and PGL-D-CHyA disc scaffolds was studied to further elucidate the effect of the polymers on the growth and proliferation of hMSCs (Figure 4.10A). LDH measurements from CHyA disc scaffolds was used as a control for comparing the effect of including a 3D printed geometry as CHyA scaffolds have been showed to be an excellent biocompatible material for cell growth^{132, 499}. No significant increase of LDH was detected for PCL37 and PCL-50-CHyA disc scaffolds, as a toxic effect on cellular growth would have resulted in an increase of LDH release when compared to CHyA scaffolds. No significant increase in the release of LDH into the cell culture supernatant was observed across the three time points under investigation. The inclusion of PGL did not led to any toxic effect on cells therefore proving a sufficient biocompatibility profile similar to that observed for the CHyA and PCL-CHyA disc control scaffolds. The decrease seen in day 7 and 14 in PGL-D-CHyA disc scaffolds compared to CHyA scaffolds can be explained due to the lower amount of cells able to attach and proliferate in PGL-D-CHyA disc scaffolds as seen in cell metabolic activity data as well as DNA content (Figure 4.9A, Figure 4.10B). DNA content measured from scaffolds was also evaluated to further confirm the presence of hMSCs in the constructs as well as to determine the ability of PGL to support the growth of hMSCs (Figure 4.10B).

PGL-D-CHyA disc scaffolds showed a lower DNA content in comparison to CHyA and PCL-CHyA scaffolds, but no significant differences were observed between day 1, 7 and 14, proving again a good biocompatibility profile of PGL-D for supporting the growth of hMSCs.

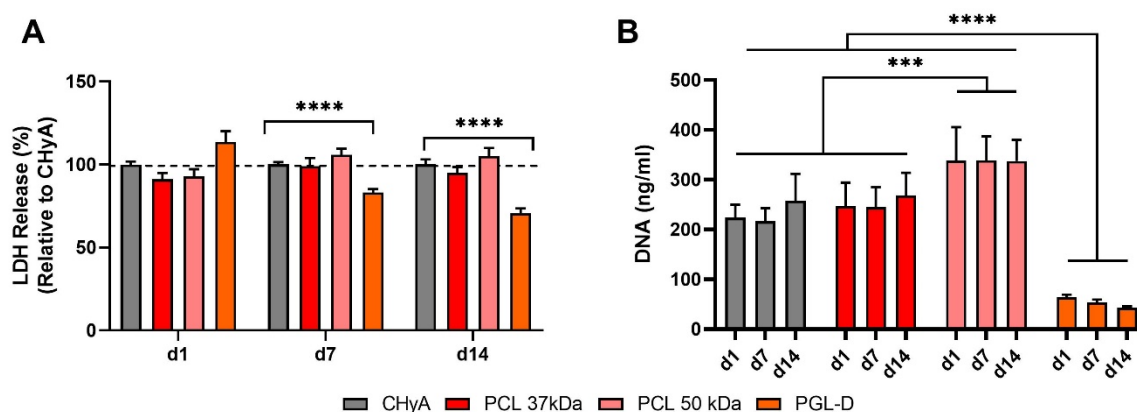


Figure 4. 10. LDH release and DNA concentration of MSCs seeded onto CHyA, PCL-37-CHyA, PCL-50-CHyA and PGL-D-CHyA disc scaffolds. (A) LDH release measured in cell culture supernatant normalised to the secretion measured on CHyA scaffolds at day 1, 7 and 14 of culture. (B) DNA quantification measured at day 1, 7 and 14 of culture (n=3, ***p<0.001, ****p<0.0001).

Finally, in order to confirm the biocompatibility of PGL-D and the presence of hMSCs in PGL-D-CHyA disc scaffolds, a modified version of LiveDead assay using DAPI was carried out (Figure 4.11). Ethidium homodimer-1 (shown in red) was used to stain dead cells while DAPI (blue) was used to stain viable cells. As shown in Figure 4.11, cells stained with DAPI were visible across all scaffold groups including PGL-D-CHyA disc scaffolds. CHyA and PCL-37-CHyA scaffolds showed the higher amount of viable cells visible in culture, with little presence of dead cells. A reduction in the number of viable cells was observed for PCL-50-CHyA and PGL-D-CHyA scaffolds but no dead cells were visualised. The presence of viable cells together with the low presence of cells stained with ethidium homodimer-1, which acts as a dead compound staining, proved PGL-D's biocompatibility, although it was clear that its ability to support the growth of hMSCs lower when compared to CHyA and PCL-37-CHyA disc scaffolds.

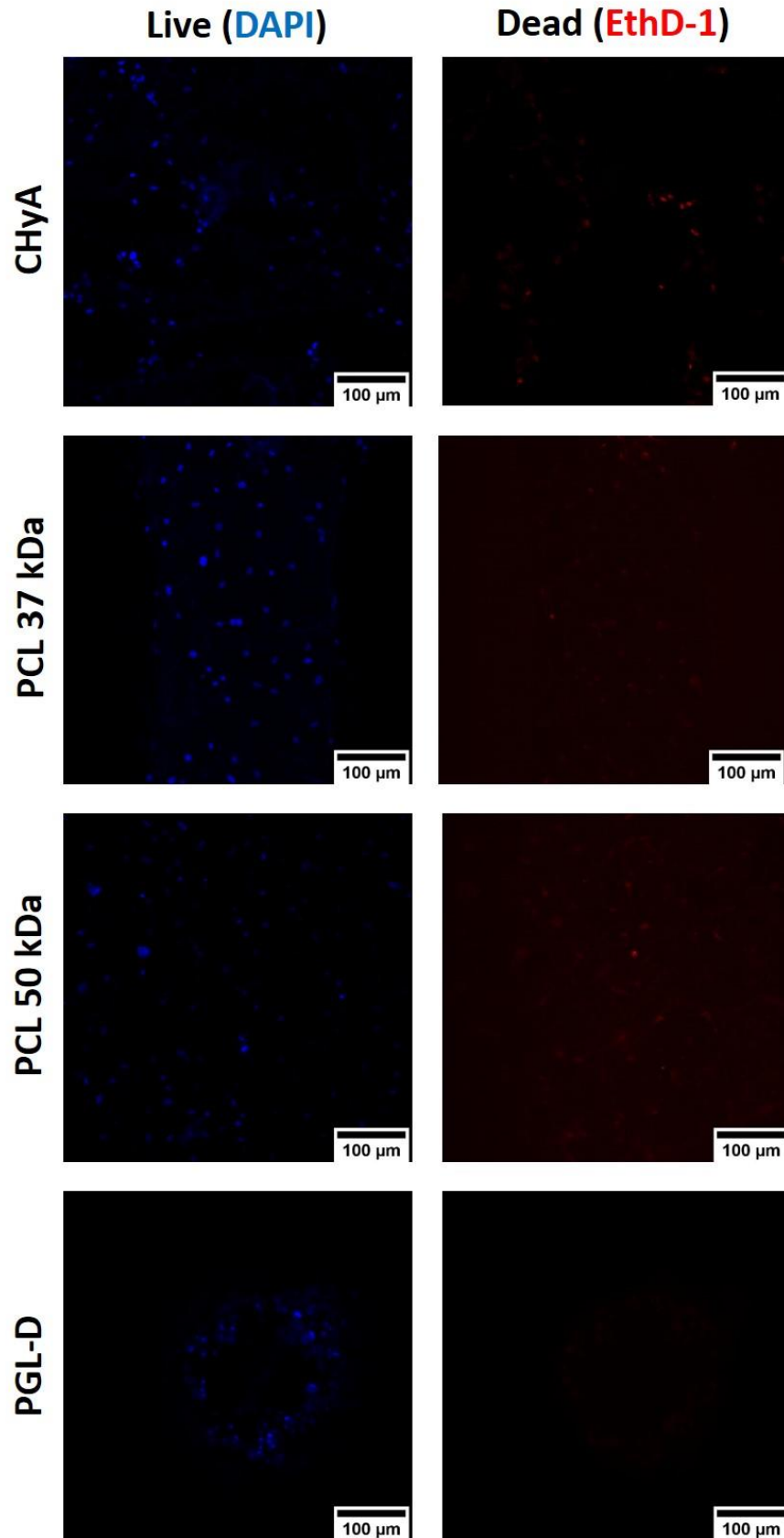


Figure 4. 11. LiveDead stain using DAPI and ethidium homodimer-1 of hMSCs grown on CHyA, PCL-37-CHyA, PCL-50-CHyA and PGL-D-CHyA disc scaffolds seeded with human MSCs (n=2).

4.4 **Discussion**

A wide range of technologies exist for the manufacture of TE scaffolds, on which cells can attach, proliferate and grow including solvent-casting, particulate-leaching or freeze-drying among others⁵⁰⁰. However, the above mentioned techniques suffer from several limitations such as imprecise pore size and geometry, formation of undesired films layers reducing cell attachment, poor mechanical integrity and release of toxic products⁵⁰¹. 3D printing techniques have recently gained popularity in tissue engineering applications to manufacture novel scaffolds with enhanced properties. Several 3D printing techniques have been explored including sterolithography, selective laser sintering, additive manufacturing and fused deposition modelling- the latest two are commonly referred to as 3D printing^{179, 500}. Although 3D printing approaches undoubtedly hold potential as manufacturing techniques in regenerative medicine, they are severely limited by the biomaterials that can be used⁵⁰². Extrusion-based additive manufacturing of 3D polymeric scaffolds represents a cheap technology that allows for the development of complex geometries and porous structures using thermoplastic materials. However, high temperatures needed in the 3D printing processes as well as the high price of raw printable materials have left PCL as the most commonly used material in 3D printing applications for tissue engineering^{162, 179, 503, 504}. However, the use of thermoplastics such as PCL requires high temperature and pressure for printing²³. PCL-based scaffolds also suffer from low biological activity for tissue regeneration applications and the hydrophobicity of 3D printed polymers can result in poor cell adhesion⁵⁰⁰. Moreover, PCL does not easily lend itself for advanced post-processing modifications which could increase its performance as a biomaterial without the need for further combination into composite scaffolds⁵⁰⁵. There is consequently a need of novel polymer biomaterials that can be used to 3D print scaffolds with tunable properties including controlled geometry, porosity and increased potential for cell attachment and proliferation. Herein, we evaluated the potential of PGL as novel, printable biomaterial.

PGL is an aliphatic polyester that has been recently used in tissue engineering applications either to manufacture electrospun scaffolds or biofilms^{173, 485, 486, 506}. PGL is a non-toxic material exhibiting a low melting point, which makes it an ideal candidate for extrusion-based 3D printing applications. Moreover, the backbone of the polymer contains alkene bonds which enable the introduction of post-synthesis modifications

to the main chain of the polymer, which could provide enhanced properties in comparison to commonly used materials such as PCL^{171, 485}. Herein, we have demonstrated the feasibility of 3D printing PGL to achieve well-defined geometries and mechanically robust scaffolds using low printing temperatures in the range of 70°C, which allows for its processing using low-cost 3D printers. This is the first report to the author's knowledge of PGL being employed as a 3D printable biomaterial. Previous tissue engineering work on PGL involved the manufacture of PGL scaffold using electrospinning resulting in scaffolds with excellent biocompatibility¹⁷³. However, the manufacturing process involves the use of solvents which can lead to cell toxicity if not processed adequately, resulting in poor cell infiltration. The use of electrospinning can also result in weaker mechanical properties with low strength and stiffness in comparison to other manufacturing techniques²⁰⁹. Moreover, creation of defined geometries with electrospinning is difficult in comparison to 3D printing approaches⁵⁰⁷.

From the four different techniques used to synthesize the PGL starting materials, only PGL-D was able to produce scaffolds suitable for further biocompatibility testing. The low molecular weight found in PGL-C made it unsuitable for 3D printing precise geometries. PGL-B resulted in an inconsistent print due to the wide range of molecular weights present in the synthesised polymer as observed from the GPC (Figure 4.2, Figure 4.4A). On the other hand, both PGL-A and PGL-D were successfully 3D printed maintaining a consistent print through different printing batches as seen by the distribution of the weight of the 3D printed constructs (Figure 4.3A). Both showed a similar molecular weight and polydispersity, but PGL-D was selected as a superior polymer for 3D printing over PGL-A based on the manufacturing of better-defined constructs. The superior capacity for 3D printing with PGL-D versus the other types of PGL aligns with literature findings used to manufacture scaffolds using the same synthesizing parameters as used in this Chapter for PGL-D^{484, 485, 490, 495, 506}. This translated into a reproducible synthesis process of PGL with a uniform molecular weight distribution that further led to consistent mechanical properties and 3D printing process.

Currently, PCL is the most commonly used polymer in additive manufacturing approaches for regenerative scaffolds because of two key factors: (i) its already an

FDA-approved biomaterial and (ii) the ease of 3D printing^{508, 509}. FDA approval granted for PCL based devices has resulted in easy and cheap commercial availability of the material¹⁶². On the other hand, PGL might hold promise in overcoming current PCL limitations regarding the difficulty of post-processing modifications in order to improve cell attachment and poor cell growth present in synthetic polymers when are used as standalone materials⁵¹⁰. PGL is a promising alternative to PCL due to its lower melting point, allowing the use of simpler printers that do not need to reach high temperatures. The low melting point of PGL allows for 3D printing at low temperatures similar to PCL, which ranges between 55 and 60°C. This is a great advantage in terms of the printer used, as other materials need higher temperatures and therefore more complex equipment is needed. For instance, the PLGA melting point of 262°C results in the need for combination with solvents or other polymer blends to achieve realistic printing temperatures⁵¹¹⁻⁵¹⁴. Other polymers that require high temperatures over 200°C and complicate the 3D printing process are polyether ether ketone (PEEK), polyethylene oxide terephthalate (PEOT), polybutylene terephthalate (PBT) and poly(vinyl alcohol) (PVA). PGL-D could be 3D printed with high precision to produce defined fibres comparable to the widely used PCL. The mechanical properties were significantly different (1.67 ± 0.38 MPa) from PCL 37 kDa (3.18 ± 0.25 MPa) but show no significant differences compared to PCL 50 kDa (2.15 ± 0.494). This highlights its versatility to be used in applications that do not require high mechanical strength as PCL 37 kDa⁵¹⁵. Moreover, it was demonstrated that PGL-D retained similar thermal properties (melting point of 45°C) as well as crystallinity after the 3D printing process when compared to previous findings compared to freshly synthesised polymer⁴⁹⁵. Therefore, PGL scaffolds can be manufactured using 3D printing without affecting the thermal properties of the polymer and once printed does not introduce changes in the polymer characteristics. PGL's printability is not the only advantage of this material, as its unique chemistry allows for the immobilisation of bioactive cues and cross-linking for post-printing modifications. Although this has been successfully achieved in electrospun PGL fibres, further work is need for its application in 3D printed PGL constructs¹⁷³.

PCL and PGL discs were combined with CHyA scaffolds to evaluate its biocompatibility. At the same time, this was done to evaluate its potential use as a supporting mechanical material for the development of tubular tracheal substitutes,

which is further investigated in Chapter 5. PGL is an aliphatic polyester and is highly hydrophobic, which has clear implications in its use as a biocompatible material suitable to support cell attachment and proliferation⁵¹⁶. In contrast, PCL is a synthetic polymer obtained via ring from ϵ -caprolactone, an aliphatic polyester derived from fossil carbon⁵¹⁷. 3D printed PGL, as PCL, has porosity at the macro level, not the micro scale required for cell attachment. Suitable collagen infiltration within the 3D printed structure was seen in toluidine blue stained images of the discs and SEM images (Figure 4.7, Figure 4.8). The microarchitecture of the collagen network was further maintained upon polymer incorporation (Figure 4.7, Figure 4.8). PGL-D-CHyA disc scaffold's porosity was around 150 μm showing no significant difference to CHyA alone and PCL-CHyA constructs. These results are similar to previous porosity findings for collagen scaffolds, which have been shown to promote cellular growth⁵¹⁸. To overcome PCL's hydrophobicity, collagen sponges have been successfully incorporated into PCL 3D printed scaffolds maintaining the collagen's porosity network with excellent biocompatibility properties for the growth of chondrocytes²²¹. Similar studies have been carried out using 3D printed PLA scaffolds decorated with collagen and citrate-hydroxyapatite to promote cellular growth while exhibiting excellent compressive strength⁵¹⁹. The optimal incorporation of natural materials (CHyA) into a 3D printed made of PGL with similar results to PCL demonstrates PGL could be an alternative to other polymers used to increase the mechanical properties of collagen-based scaffolds for tissue engineering applications such as PLA and PCL^{520, 521}.

While similar collagen infiltration was seen for PCL-CHyA and PGL-D-CHyA disc scaffolds, differences in mechanical strength and NaOH sensitivity were observed when reinforcing scaffolds with PGL compared to previous findings using PCL⁵²². This different material behaviour might explain the lower cell presence in PGL-CHyA disc scaffolds, highlighting the effect of the polymer used for the reinforcement. Moreover, it was demonstrated that PGL constructs had a higher crystallinity in comparison to PCL (Figure 4.4C). This could be attributed to a higher degree of molecular freedom observed for macrolactone-derived polyesters allowing for better chain alignment and therefore crystal nucleation. Different crystallinity was also observed for the different PGL polymers synthesised, with PGL-B showing higher crystallinity versus PGL-B and PGL-D. This increase in crystallinity is a consequence of the lower molecular weight of PGL-B in comparison to PGL-A and PGL-C, previously observed for other polymeric

systems. It is important to note that the crystallinity of a polymer plays an important role in determining its biocompatibility and potential for cellular growth. It has been described how gas diffusivity is decreased when the polymers crystallinity is increased, reducing the quantity of gas entering cells⁵²³. This can result in lower cellular growths, which could help explain the reduced cellular presence on PGL-D-CHyA disc scaffolds. Furthermore, PGL and other polymacrolactones reported high hydrophobicity which may be a factor in this reduced attachment^{173, 483, 490, 516}. It was hypothesised that the hydrophobic nature of PGL played an important role in the seeding process of the scaffolds. This resulted in lower cellular attachment as measured by the cell metabolic activity and DNA content over the culture period, as the 3D printing design used offered a significant amount of polymer surface area for cell attachment (Figure 4.9, Figure 4.10B). Interestingly, PGL-based films exhibited a hydrophobic behaviour without further post-processing, which was only improved when surface functionalisation was introduced. Upon film decoration with poly(acrylic acid) (PAA), PGL films were able to absorb water droplets within 1 min, showing a change of surface hydrophilicity in comparison to non-decorated films⁵⁰⁶. Recent studies have also shown that PGL-based electrospun fibres had a contact angle of 110.5° during wettability studies, which is greater in comparison to that reported for PCL, at $101 \pm 3^\circ$ ⁵²⁴. After functionalisation of PGL with amino groups, an increased hydrophilicity was detected showing a reduction of the water contact angle to $86^\circ \pm 1^\circ$ ¹⁷⁰. Moreover, nanowire-based PCL biomaterials were shown to exhibit a contact angle of 80° , which was drastically reduced upon incorporation of collagen into the system, changing the biomaterial's hydrophobicity⁵²⁵. Interestingly, LDH release from PGL-D-CHyA disc scaffolds did not show significant increases in comparison to CHyA scaffolds. Metabolic activity as well as the DNA content measured on the PGL-D-CHyA disc scaffolds did not decrease overtime (Figure 4.9, Figure 4.10B). The presence of hMSCs on the scaffolds was further confirmed by DAPI staining (Figure 11). Taken this together, it was hypothesised that PGL shows promising properties as a printable biomaterial that supports the growth and maintenance of those cells that were able to attach, but increasing initial cell attachment during seeding should be further examined. Increasing the distance between PGL extruded fibres during the 3D printing process could lead to a greater surface area of CHyA exposed and available for cellular attachment overcoming the effect of the polymers hydrophobicity. Other possibilities to overcome this problem are the combination of PGL with other synthetic

polymers to develop copolymers as previously reported in manufacturing PGL and PCL-based films, albeit the wettability and biocompatibility of this films remains to be investigated^{526, 527}. Swelling of the polymer was not investigated throughout this thesis, but should be consider in future work to better elucidate how polymer swelling in PGL samples might affect the freeze-drying process and combination with collagen-based scaffolds with the consequent effect in cell attachment.

Further functionalisation of PGL 3D printed matrices is needed to efficiently address its hydrophobicity properties and detrimental effects in cell seeding and proliferation. One initial aim of this PhD thesis was to functionalize 3D printed constructs before cell seeding in order to overcome the hydrophobicity of the polymer. Due to the COVID-19 pandemic and its effect not only in the work carried out in this thesis but also in the chemistry work carried in collaboration with the Chemistry Department in RCSI University of Medicine and Health Sciences, further functionalisation of PGL constructs was not able to be investigated.

4.5 Conclusion

In previous Chapters of this thesis, the versatility of CHyA-B scaffolds for tissue engineering applications of the airway, specifically of the tracheobronchial region was demonstrated. In this Chapter, the possibility of using PGL as a novel biomaterial to develop 3D printed constructs that can be coupled with CHyA scaffolds for tissue engineering applications was investigated. The following conclusions can be obtained from this Chapter 3:

- PGL can be successfully employed to manufacture 3D printed scaffolds with similar properties to other commonly used polymers such as PCL.
- PGL constructs combined with CHyA-B indicated that the presence of PGL had a detrimental impact on initial hMSC cell attachment but promising support for cell growth once attached.
- PGL hydrophobicity critically affects cell attachment and further functionalisation of the surface to encourage cell attachment will be needed for further applications to enhance the biomaterial's performance.

Chapter 5

Development of Polymer-reinforced CHyA-B Tubular Scaffolds for Tracheal Tissue Engineering and Repair

5.1 Introduction

Tracheal damage, often a life-threatening condition, is commonly associated with the narrowing, weakening and discontinuity of the conductive part of the lower respiratory tract³⁵. These defects arise from a variety of pathologies including traumatic injury, several types of cancer, tracheobronchiomalacia (TBM) and due to prolonged intubation time – especially arising from COVID-19 complications recently^{255, 256, 262, 528}. Tracheal defects short in length undergo thoracic surgery and end-to-end anastomosis, but this is not possible for extensive tracheal damage exceeding 5 cm in adults and 2 cm in children⁴⁵³. In such cases, patients are treated with tracheoplasty, artificial reconstruction and/or novel prostheses³⁵. However, these approaches usually lead to complications including weakening of the implant over time and tissue granulation, leaving tracheal replacement as an unmet clinical need for which novel approaches to support effective restoration and regeneration are urgently needed^{264, 296, 529}.

Several approaches have been investigated to regenerate tracheal tissues. Both solid and porous prostheses have been employed as a way of solving this surgical challenge with mixed outcomes, usually resulting in tissue granulation and material infection with high mortality rates^{72, 530, 531}. Porous prostheses were later developed and its clinical use gave rise to the widely used current tracheal stents, which aim at maintaining airway patency and avoiding tracheal collapse rather than encouraging tissue regeneration²⁷⁹. Stenting approaches for airway obstruction are mainly made of silicone and metals such as Ultraflex and Wallstent⁵³². Silicone stents are manufactured using two main designs: Y-shape and straight²⁷⁹. On the other hand, metallic airway stents present hollow tubular designs that can be self-expanding or balloon-dilated⁵³³. Efforts have recently shifted towards the use of tissue-engineered (TE) strategies that could maintain tracheal patency while promoting the regeneration of tracheal tissues around the defected site⁷³. A wide range of biomaterials, as well as different manufacturing techniques including electrospinning, decellularisation of grafts, casting and 3D printing, have provided crucial knowledge for developing next generation tracheal substitutes: mechanical strength alongside with excellent biocompatibility properties and support of vascularization^{97, 158, 284, 453}. 3D printing is a popular and innovative tool used in TE applications for its versatility, affordable price and accessibility. It further allows the development of 3D printed scaffolds using a wide

range of biocompatible materials and allows easy control of the printed geometry and fibre diameter^{179, 505}. However, there is need for the development of novel advanced printable materials that allow easy introduction of post-processing modifications to overcome limitations of current synthetic 3D printed materials that can suffer from reduced cell attachment and growth when used as standalone materials⁵³⁴.

In spite of the wide range of techniques investigated for this purpose, including both TE and non-TE approaches, none to-date have succeeded in supporting full tracheal restoration for three main reasons;

(i) Sufficient mechanical properties: a lack of appropriate mechanical properties resulting in tracheal stenosis, implant weakening over time and therefore implant failure²⁰⁶. For example, following implantation of collagen and polycaprolactone (PCL) tracheal scaffolds in mice, regeneration of tracheal tissues with mucosal lining was observed. However, the lack of appropriate mechanical strength resulted in airway collapse and deformation hindering further clinical translation^{302, 535}.

(ii) Supporting enough blood vessel formation: vascularisation is key in graft success by avoiding tissue necrosis and providing nutrients to support the formation of new cartilage and epithelium, which is sometimes not fully considered during the development of tracheal substitutes²⁶⁷. Pre-vascularisation of tissue-engineered trachea (TET) scaffolds has been demonstrated to support faster epithelialisation and guaranteed long-term trachea reconstruction⁴⁶⁰.

(iii) Facilitating maturation of epithelium: the formation of a fully functional and mature respiratory epithelium in the inner lumen of the substitute is needed for mucociliary clearance and prevention of bacterial infection which will critically endanger implant performance²⁶⁶.

Currently, there is also limited pre-clinical assessment and prototyping of tracheal scaffolds, which hinders further development and clinical translation of TET approaches²². This is addressed with the development of tracheal tubular constructs proposed in this Chapter. Several techniques including decellularised and electrospun scaffolds have been implanted in different animal models with poor success⁷². More recently, 3D printing has caught the attention of researchers for the development of tracheal scaffolds that could potentially be used as tracheal substitutes^{215, 453, 536}. 3D

printing technologies offer the possibility of rapid fabrication of custom-made scaffolds with tunable architecture, mechanical properties as well as degradation rates^{210, 211}. For example, 3D printed PCL scaffolds designed for trachea restoration have been already develop and combined with collagen. Although the porosity of the biomimetic scaffold showed to be suitable for cell growth, poor mechanical, lack of proper *in vitro* characterisation prior to implantation and a small number of specimens used lead to mix outcomes with reports of granulation and no long term assessment of the implants²²¹. In this Chapter, 3D printing technologies were combined with collagen and hyaluronic acid bilayered (CHyA-B) scaffolds for the development of novel tubular scaffolds for tracheal tissue engineering. Extensive work has been carried out in the *in vitro* characterisation of 3D printed tracheal scaffolds and a number have moved onto *in vivo* evaluation including rabbit, goat and rat models^{76, 215, 219, 453, 537}. 3D printed constructs have been shown to be particularly useful in the paediatric setting for patients and some cases of adults suffering from TBM, demonstrating the feasibility of 3D printed constructs as a promising solution for the treatment of tracheal defects^{214, 316, 317}.

Therefore, 3D printing of polyglycolide (PGL) optimised in Chapter 4 was further challenge for the development of tubular geometries to act as the reinforcing backbone for TE scaffolds aimed at tracheal regeneration and restoration. PGL has been already used for the development of biocompatible scaffolds using electrospinning techniques and has also been proven as an excellent candidate for 3D printing applications in the previous Chapter^{170, 173, 485}. Moreover, PGL was harnessed for its application in tracheal TE scaffolds as post-processing modifications, to tune its properties for biocompatibility and cellular growth, can easily be introduced in comparison to PCL which is commonly used for the development of tracheal scaffolds^{156, 170, 219, 538}. To overcome previous findings on low cellular attachment of PGL-based scaffolds, CHyA-B scaffolds previously reported as an excellent platform for respiratory TE applications (Chapters 3 and 4) were further combined with the 3D printed tubular structure. In order to overcome current limitations in the development of tracheal scaffolds and their low clinical translation due to mismatched properties²², an extensive *in vitro* characterisation of the proposed tubular scaffolds was implemented including mechanical testing, porosity and ultrastructure examination and biocompatibility studies to evaluate the formation of a respiratory epithelium in the scaffolds.

5.1.1 Aims and Objectives

The main aim of this Chapter was to manufacture tubular 3D printed scaffolds using PCL and PGL characterised in Chapter 4 in combination with CHyA-B scaffolds used in Chapter 2 and 3 to assess its potential as scaffolds aiming at tracheal repair and regeneration. 3D printed tubular scaffolds were characterised and a method to enable cell seeding of the inner lumen of the constructs using epithelial cells was developed. These prototypes were explored as proof of concept for the generation of a mature epithelium on the 3D printed tubular scaffolds. The specific objectives for this Chapter were:

- To develop and characterise tubular 3D printed geometries using both PCL and PGL in combination with CHyA-B scaffolds.
- To optimise epithelial seeding using PCL-based tubular scaffolds to establish a process for evaluation of cellular attachment, viability and epithelialisation.
- To explore the potential of PGL as an alternative polymer to PCL in tubular constructs for tracheal tissue engineering and compare the biocompatibility and epithelialisation profile for both constructs.

5.2 Materials and Methods

5.2.1 Fabrication of PCL- and PGL-CHyA-B Scaffolds

5.2.1.1 Polyglobalide (PGL) Synthesis

This work was carried out with the assistance of Dr. Ruairí Brannigan, Polymer Chemistry and Biopolymer Labs, RCSI University of Medicine and Health Sciences.

Globalide (GL) was used as the monomer for obtaining PGL. Synthesised PGL for this Chapter corresponds to PGL-D from Chapter 4. For its synthesis, 15 g of GL and NOVOZYM 435 (Candida Antartica Lipase B immobilized on cross-linked polyacrylate beads; Novozymes A/S, Denmark) were weighted and dried for 24 h under vacuum at 50°C. The lipase enzyme was added to the monomer at a 6% wt ratio into a two-neck round bottom flask and 30 ml of Toluene (Toluene, 99.85%, Extra Dry over Molecular Sieve, AcroSeal™, ACROS Organics™; Fisher Scientific) were added. The reaction flask was purged with nitrogen and placed in an oil bath at 60°C under magnetic stirring for 4 h. Dichloromethane (DCM) was added to the solution to reduce system's viscosity and was filtered using a Büchner funnel with vacuum applied to inactivate the enzyme and removed it from the polymer solution. After filtration, DCM was evaporated from the solution using a rotary evaporator. Finally, the solution containing the polymer was precipitated using 600 ml of cold methanol under magnetic stirring. The obtained polymer was dried overnight in a vacuum chamber to remove any residual methanol. PCL with a molecular weight of 37 kDa was obtained from VWR (VWR, Ballycoolin, Ireland).

5.2.1.2 Scaffold Design and 3D Printing

Polymer constructs were 3D printed using a Biobot1 (Allevi, USA) printer. Metal syringes were rinse twice in chlorophorm (99+% ACROS Organics™) and 70% v/v ethanol prior to use to remove any residual polymer. Python was used to generate the g-codes needed for printing the geometries of the 3D constructs. Two geometries were 3D printed: a tubular and a c-shape construct (Figure 5.1). Both designs had the inner and outer circumferences connected by 5 spokes for achieving mechanical integrity of the printed constructs. PCL constructs were printed at 100°C using a pressure of 90 PSI for extrusion, while PGL constructs were printed also at 100°C but using a

pressure equal to 55 PSI using a 25G metal needle (Inner diameter 0.26 mm, Micron-S Fisnar; ECT Adhesive & Dispensing Solutions, Dublin, Ireland).

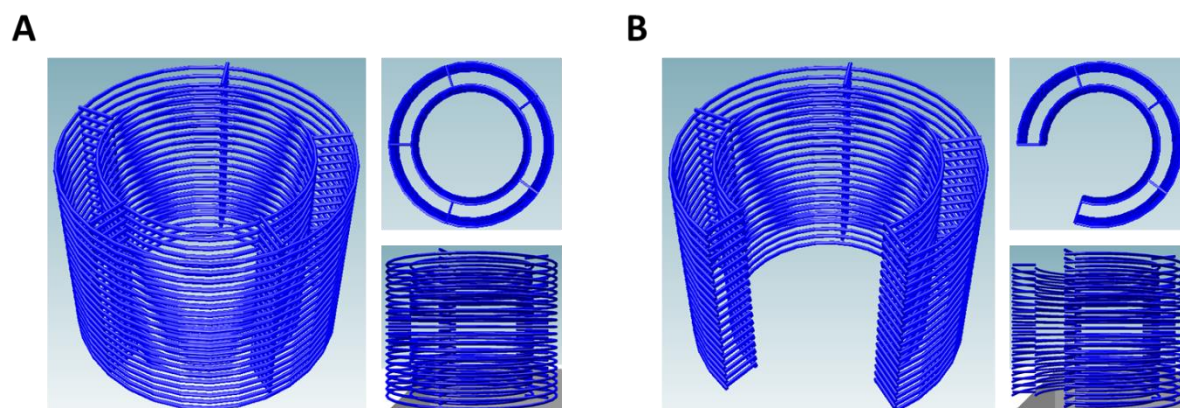


Figure 5. 1. Designs used to 3D print tubular constructs of PCL and PGL, consisting of 40 layers (0.25 mm each) of 4.8 and 6.6 mm inner and outer radius interconnected with 5 spokes in each layer. (A) Schematic view of tubular design and (B) c-shaped design comprising 288°.

5.2.1.3 Preparation of Collagen and Hyaluronic Acid (CHyA) Slurry and Films

Collagen and hyaluronic acid (CHyA) films were prepared as previously described in Chapter 2, Section 2.2.2.1 using a suspension of 0.5% w/v type I bovine tendon collagen and 0.044% w/v hyaluronic acid (HyA) in 0.5 M acetic acid (AcOH). CHyA slurry was blended at 4°C and 15,000 rpm for 3.5 h, after which it was degassed to remove air bubbles introduced during the homogenisation process. The CHyA slurry was pipetted onto 6x6 cm² polytetrafluoroethylene (PTFE) plate to produce thin transparent CHyA films.

5.2.1.4 Freeze-drying of PCL and PGL-CHyA-B Scaffolds

PCL and PGL 3D printed backbones were combined with CHyA-B scaffolds to manufacture PCL- and PGL-CHyA-B tubular and c-shape scaffolds. PCL and PGL 3D printed tubular constructs were treated for 4 h with 3 M NaOH solution in Dulbecco's phosphate-buffered saline (DPBS) to reduce hydrophobicity and increase collagen infiltration. Polymer-reinforced CHyA-B scaffolds were manufactured using a customised stainless-metal mould shown in Figure 5.2 that consisted of 16 pegs of 26 mm in height and 7.8 mm radius in the bottom plate. The top plate consisted of the respective 16 holes 14 mm wide to allow for the development of tubular scaffolds. CHyA films were cut into 26 x 30 mm strips, rehydrated for 2 h in 0.5 M AcOH and wrapped around the pegs of the mould. Films were left overnight under an airflow in a

fume hood to allow dehydration of the films and attachment to the pegs. The following day, 1 ml of CHyA slurry was pipetted into the wells of the metal mould while the 3D printed constructs were gently dropped into the slurry and allowed to sink. The combination was incubated for 2 h at room temperature (RT) to allow for film rehydration and combination with the CHyA slurry, and the scaffolds were thereafter freeze-dried as described in Chapter 2, Section 2.2.2.2.

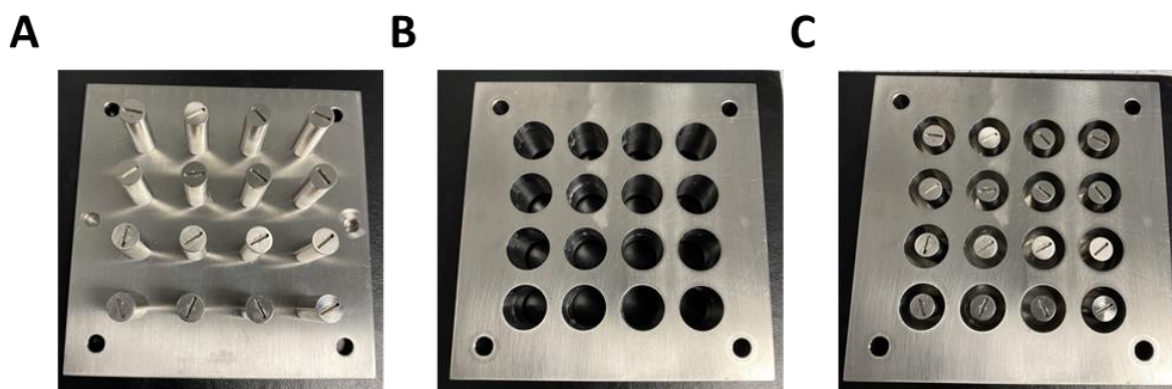


Figure 5. 2. Custom stainless-steel mould use to freeze-dry tubular polymer-reinforced CHyA scaffolds (A) Upper plate, (B) lower plate and (C) mounted mould.

5.2.1.5 Cross-linking and Sterilisation

Prior to cell seeding and evaluation of biocompatibility, the scaffolds were sterilised in an ethylene oxide Anprolene AN74 Series gas sterilizer and chemically cross-linked as described in Section 2.2.2.3 using 6mM of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) per gram of collagen and N-Hydroxysuccinimide (NHS) in a 5:2 molar ratio.

5.2.2 Characterisation of PCL- and PGL-CHyA-B Scaffolds

5.2.2.1 Mechanical Characterisation

PCL- and PGL-CHyA-B tubular and c-shape scaffolds were mechanically characterised with a mechanical testing machine Z050 (Zwick Roell, Germany). In order to evaluate the strength of the different 3D printed designs and the effect of each polymer used, the scaffolds were tested using longitudinal and radial compression with a 50 N load cell and 15% strain. The longitudinal compressive modulus was then calculated using the stress-strain curves as described in Section 4.2.4.2. Due to the change in surface area while performing radial compression, the maximum peak load in N was used to measure the construct performance. For radial compression of c-

shape constructs, two positions were employed: the opening facing down parallel to the direction of the force applied or facing to the side, perpendicular to the applied force. Moreover, to further assess the strength of the scaffolds, they were subjected to 15% strain at 20 cycles per minute (0.33 Hz) for a total amount of 250 cycles in order to mimic the fast breathing rate observed in humans. Differences between cycle 1 and 250 and between the different designs and polymers were then evaluated. For c-shape designs, the opening was oriented to the side, perpendicular to the direction of the applied force.

5.2.2.2 Scaffold Ultrastructure Characterisation

To evaluate the porosity of PCL- and PGL-CHyA-B tubular scaffolds and the effect of the tubular customised mould, pore size analysis was carried out as described in Section 4.2.7 using a JB[®]-4 Embedding Kit and stained using 0.5% v/v toluidine blue solution in distilled H₂O. Moreover, collagen infiltration within the 3D printed construct was studied using scanning electron microscopy (SEM) imaging as described in Section 4.2.8.

5.2.3 Epithelialisation on PCL- and PGL-CHyA-B Tubular Scaffolds

5.2.3.1 Cell Selection and Culture Medium

The Calu-3 bronchial epithelium cell line was cultured as previously described in section 2.2.3.1. Briefly, Calu-3 cells were seeded at a density of 1×10^6 cells per T175 flask using a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% v/v foetal bovine serum (FBS). Cells were used between passage 30-39.

5.2.3.2 Seeding Density and Rotation Time Optimisation

This initial optimisation work was carried out within the team by Dr. Derek Whelan, Cryan's lab team, School of Pharmacy and Biomolecular Sciences, RCSI University of Medicine and Health Sciences.

PCL-CHyA-B tubular scaffolds were used to establish the optimal seeding density and conditions. Scaffolds were pre-warmed in 2 ml of Calu-3 medium for 15 min prior to cell seeding. Scaffolds were then placed in 15 ml Corning[®] centrifuge tubes that had been filled with melted PCL until the 12 ml mark for tight placement of scaffolds during

the seeding process. Three different seeding densities were used as shown in Table 5.1. Each scaffold was seeded with the right amount of cells for each density in 1 ml of medium and placed under rotation (0.5 rpm) at 37°C and 5% CO₂ using a Bottle/Tube Roller (Fisher Scientific). Moreover, seeding time needed for appropriate cell attachment was also studied for 1, 2 and 4 h once an optimal seeding density was obtained. After seeding time under rotation, scaffolds were transferred to a 6 well plate and were fed three times a week with 10 ml of Calu-3 medium. Seeding efficiency was evaluated using alamarBlue™ Cell Viability Reagent as previously described in section 2.2.5.1. Briefly, scaffolds were transferred to a 24 well plate and 2 ml of 1X alamarBlue™ solution was added and incubated for 2 h. Fluorescence was measured using a 545 nm excitation and 590 nm emission wavelengths in a Tecan Infinite M Plex plate reader.

Table 5. 1. Epithelial seeding densities used for tubular polymer-reinforced CHyA-B scaffolds.

	Seeding Density (cells/cm²)
Density A	5 x 10 ⁵
Density B	10 x 10 ⁵
Density C	20 x 10 ⁵

5.2.3.3 Optimisation of Epithelialisation on PCL-CHyA-B Tubular Scaffolds

Once the optimal seeding density and the seeding time under rotation were established, PCL-CHyA-B tubular scaffolds were cultured with Calu-3 cells for 10 days. Cell metabolic activity, DNA quantification and epithelial coverage of the film of the tubular scaffolds was evaluated at day 2, 7 and 10 in order to establish an optimal time to evaluate the epithelial coverage on the scaffolds. Metabolic activity from cells seeded on PCL-CHyA-B tubular scaffolds was assessed as described in Section 5.2.3.2. Seeded tubular scaffolds were processed to quantify double stranded DNA (dsDNA) as described in Section 4.2.9.4. Briefly, the film was detached from the tubular scaffold, rinsed in DPBS and submerged in 1 ml of lysis buffer and thawed three times at -80°C before analysing DNA content using a PicoGreen® dsDNA Kit. Films were also detached from the scaffold to evaluate the epithelial cell coverage of the film. Scaffolds were rinsed in DPBS and fixed using 1.5 ml of 10% v/v neutral buffered formalin solution at RT for 10 min. Following every step, films were washed

in DPBS for 3 min under rotation at 5 rpm. All following steps were performed under rotation at RT. Films were thereafter incubated with 0.1% v/v Triton X-100 in DPBS for cell permeabilisation during 5 min, followed by 1% w/v bovine serum albumin (BSA) in DPBS for 30 min. Films were stained using 1:600 phalloidin (Phalloidin–Tetramethylrhodamine B isothiocyanate) diluted in 1% v/v BSA in DPBS for 20 min followed by a 10 min incubation with 1:1000 DAPI readymade solution diluted in 1% BSA in DPBS. Films were then placed in coverglass slides (VWR) and a drop of Invitrogen™ ProLong™ Glass Antifade Mountant (BioSciences) was used to cover the films and sealed with nail polisher (Boots, Dublin, Ireland). Epithelial coverage on the films was evaluated by imaging the films with a Nikon Eclipse 90i microscope and estimating the cell coverage using FIJI-based code develop by Brenton Cavanagh (Cellular and Molecular Imaging Core, RCSI University of Medicine and Health Sciences). Images were obtained using 4X and 10X magnification, although whole film images using the 4X magnification were used for estimating the epithelial coverage. Briefly, the code calculated the area in cm² of the film by using the DAPI stain which was also able to stain the CHyA film while the area in cm² covered by cells was estimated using the phalloidin stain. Finally, the coverage of the film was obtained using Equation 5.1:

Equation 5. 1. Epithelial coverage calculation.

$$\text{Epithelial coverage (\%)} = \frac{\text{Area stained with Phalloidin (cm}^2\text{)}}{\text{Area stained with DAPI (cm}^2\text{)}} \times 100$$

5.2.3.4 Epithelialisation on PCL- and PGL-CHyA-B Tubular Scaffolds

Once the optimal days of culture for evaluating epithelialisation and cell coverage of polymer-reinforced scaffolds was established, the ability of PGL-CHyA-B tubular scaffolds was evaluated using PCL-CHyA-B tubular scaffolds as control. Tubular scaffolds were seeded as described in Section 5.2.3.2 with a seeding density of 5 x 10⁵ cells/cm². Cell metabolic activity, DNA quantification and epithelial coverage of the film were performed as reported in Section 5.2.3.3.

5.2.4 Statistical Analysis

Quantitative data obtained were analysed using Microsoft Excel and GraphPad Prism 7.0 Software. In cases of analysis between two groups, statistical difference was

assessed by two-tailed Student t-test. For multiple groups, statistical difference between groups was assessed by 2-way Analysis of Variance (ANOVA).

5.3 Results

5.3.1 3D printing of PCL and PGL Tubular and C-shaped Backbones

Tubular and c-shape geometries were 3D printed to mimic the trachea tubular structure and to account for the c-shape tracheal cartilaginous rings. Tubular and c-shape PCL and PGL constructs were successfully printed consisting of 40 layers with a 4.8 and 6.6 mm inner and outer radius interconnected with 5 spokes in each layer (Figure 5.3A,B). For PCL, printing parameters from Chapter 4 were used to provide a reproducible print of tubular and c-shape constructs at 100°C using 90 PSI. To better control the 3D geometry, the printing speed used was reduced from 280 to 260 mm/min. On the other hand, 3D printing parameters established for PGL in Chapter 4 (70°C, 55 PSI and 350 mm/min) proved to be insufficient to achieve a similar precise geometry as the one obtained with PCL. Therefore, temperature was increased to 100°C, which facilitated more accurate and reproducible printing of PGL similar to PCL (100°C, 90 PSI and 260 mm/min). The rest of the parameters for PGL remained unchanged: 55 PSI and 350 mm/min. (Figure 5.3C).

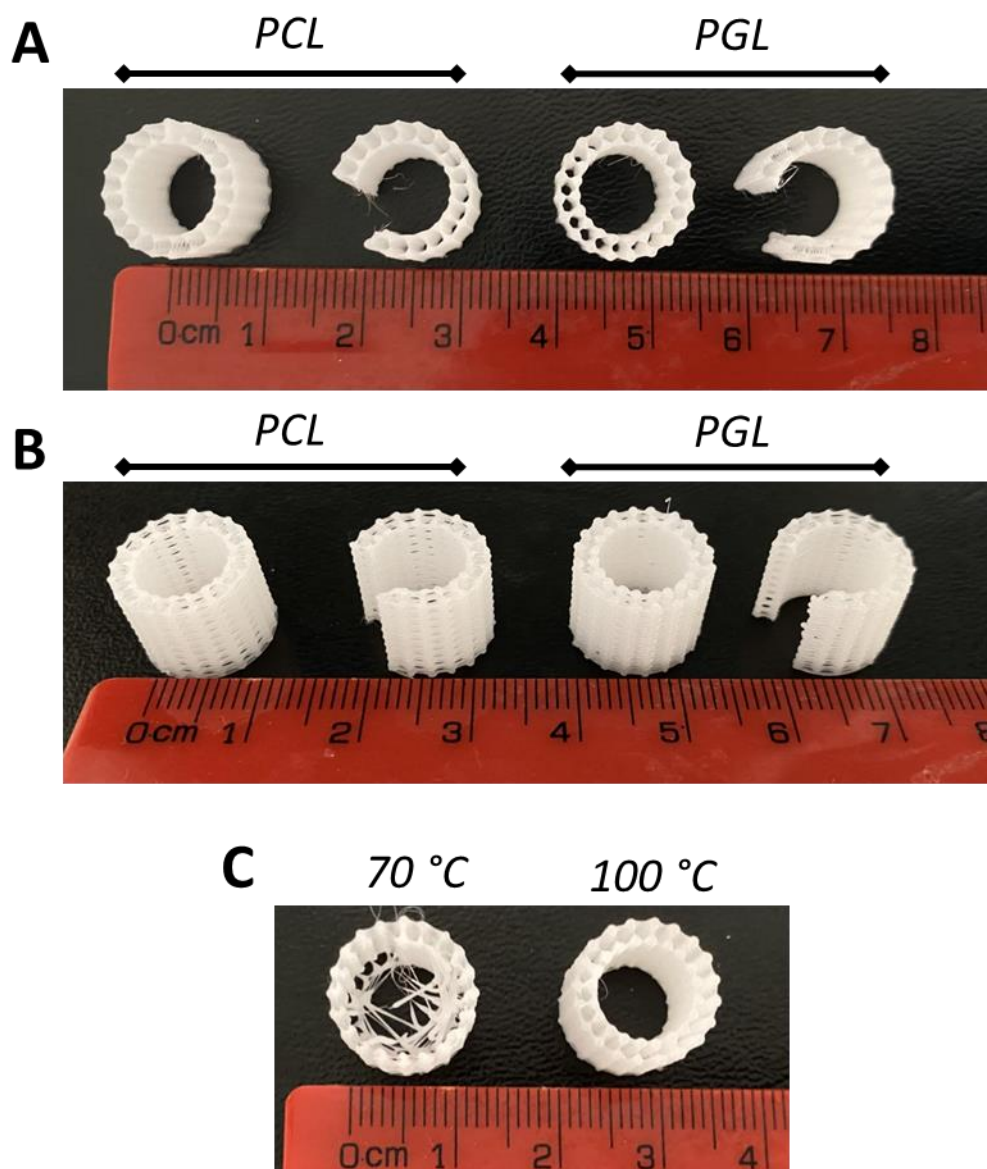


Figure 5. 3. Representative images of PCL and PGL tubular and c-shape 3D printed backbone constructs comprising 40 layers of extruded polymer of 1 cm in height (PGL was printed using 55 PSI and 70 or 100°C and an extrusion speed of 350 mm/min while PCL used 90 PSI, 100°C and 260 mm/min). (A,B) Top and lateral view of PCL and PGL 3D printed constructs. (C) PGL tubular constructs 3D printed using 70 and 100°C.

5.3.2 Mechanical Characterisation of PCL- and PGL-CHyA-B Tubular and C-shape Scaffolds

PCL and PGL 3D printed backbones were freeze-dried with CHyA-B scaffolds to develop PCL- and PGL-CHyA-B tubular and c-shape scaffolds (Figure 5.4). Longitudinal and radial compressive testing of the PCL- and PGL-CHyA-B scaffolds was performed to determine the compressive modulus of the constructs and determine any difference between the two different designs and the polymers used (Figure 5.5). The inclusion of both PCL and PGL 3D printed networks proved to very significantly increase the strength of the scaffold in comparison to non-reinforced CHyA scaffolds (Figure 5.5A). Longitudinal compression did not show significant differences between the tubular and c-shape designs employed for each polymer backbone, but PGL-CHyA-B scaffolds had a decreased compressive modulus in comparison to PCL. Similar results were obtained when performing radial testing, where PCL- and PGL-CHyA-B scaffolds, as expected, were able to increase the supported peak load compared to non-reinforced CHyA scaffolds (Figure 5.5B). Moreover, a significant decrease in the supported peak load was seen between the tubular and radial design for both polymers. Again, all PGL constructs appeared to be slightly mechanically weaker when compared to PCL scaffolds (Figure 5.5B).

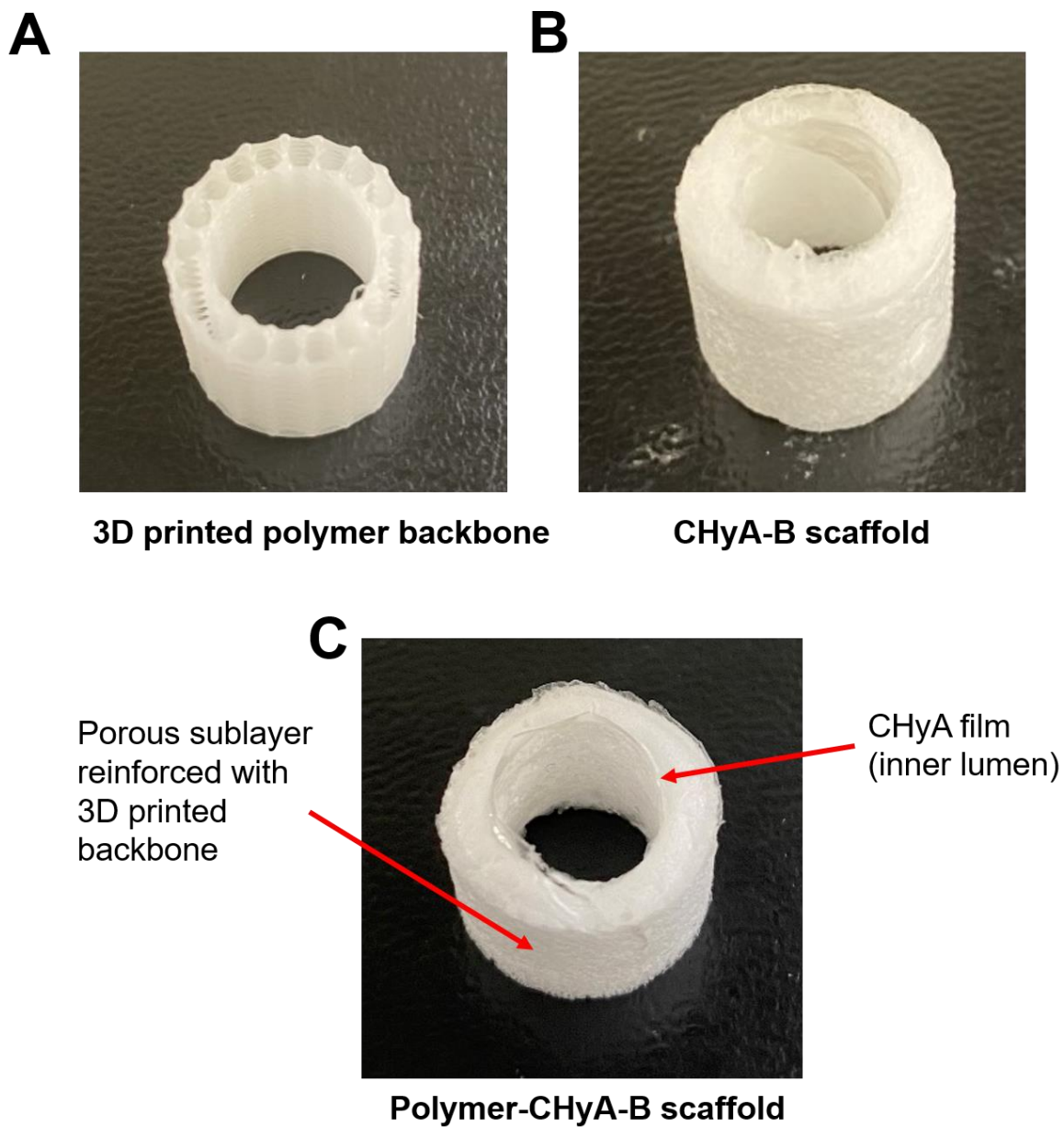


Figure 5. 4. Representative images of PCL- and PGL-CHyA-B tubular scaffolds. 3D printed tubular PCL or PGL backbones (A) were combined with CHyA-B scaffolds (B) and freeze-dried to produce PCL- and PGL-CHyA-B tubular scaffolds.

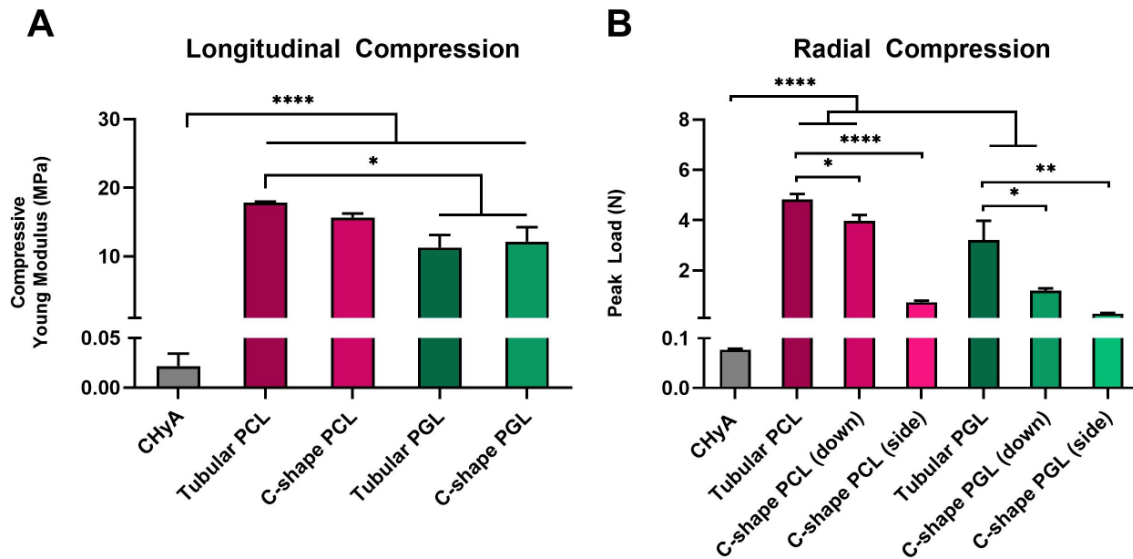


Figure 5. 5. Mechanical compression of CHyA and PCL- and PGL-CHyA-B tubular and c-shape scaffolds using a 50N load cell, 1 cycle and 15% strain. (A) Young Modulus in MPa after performing longitudinal compression and (B) peak load in N after performing radial compression. Results displayed as mean $\pm$ SEM (n=4, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Fatigue testing was employed to evaluate if the designs were able to withstand constant mechanical loading to mimic the respiration force patterns being continually exerted to tracheal tissues. All 4 scaffold types were able to withstand the cyclic loading of 250 cycles, showing no significant decrease when compared to the respective cycle 1 (Figure 5.6). Interestingly, a very significant decrease in peak load of around 4 N was observed for both PCL and PGL-CHyA-B scaffolds between the tubular and c-shape designs (p value < 0.0001). This shows the influence of the c-shape design in the scaffold radial mechanical performance. From here on, tubular designs were only used for further porosity, biocompatibility and epithelialisation assays.

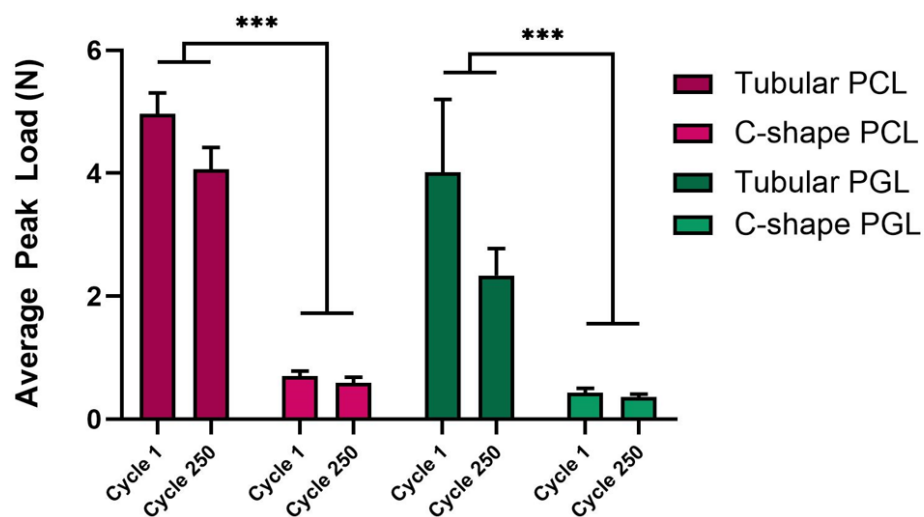


Figure 5. 6. Average peak load in N of PCL and PGL-CHyA-B tubular and c-shape tubular and c-shape scaffolds mechanically assessed under cyclic loading using a 50N load cell and 15% strain. The average peak load was measured at cycle 1 and cycle 250. Results displayed as mean $\pm$ SEM (n=4, ***p<0.001).

5.3.3 Scaffold Ultrastructure Characterisation

Pore size analysis, toluidine blue imaging and SEM imaging was carried out to ensure the CHyA slurry was effectively incorporated into the backbone of tubular PCL-CHyA-B and PGL-CHyA-B scaffolds. The CHyA slurry was effectively incorporated into the tubular designs 3D printed backbones of PCL and PGL. A similar pore morphology and distribution was observed as the CHyA-B tubular scaffolds as shown with examples of toluidine blue slide staining in Figure 5.7A-C. Pore size analysis revealed a significant increase above 150 nm in pore diameter for reinforced tubular scaffolds compared to CHyA-B alone. There was no significant difference observed between PGL- and PCL-CHyA-B scaffolds in the diameter of the collagen pores formed after freeze-drying (Figure 5.7D). SEM imaging as shown in Figure 5.8 further confirmed successful infiltration of the CHyA slurry within the 3D printed constructs.

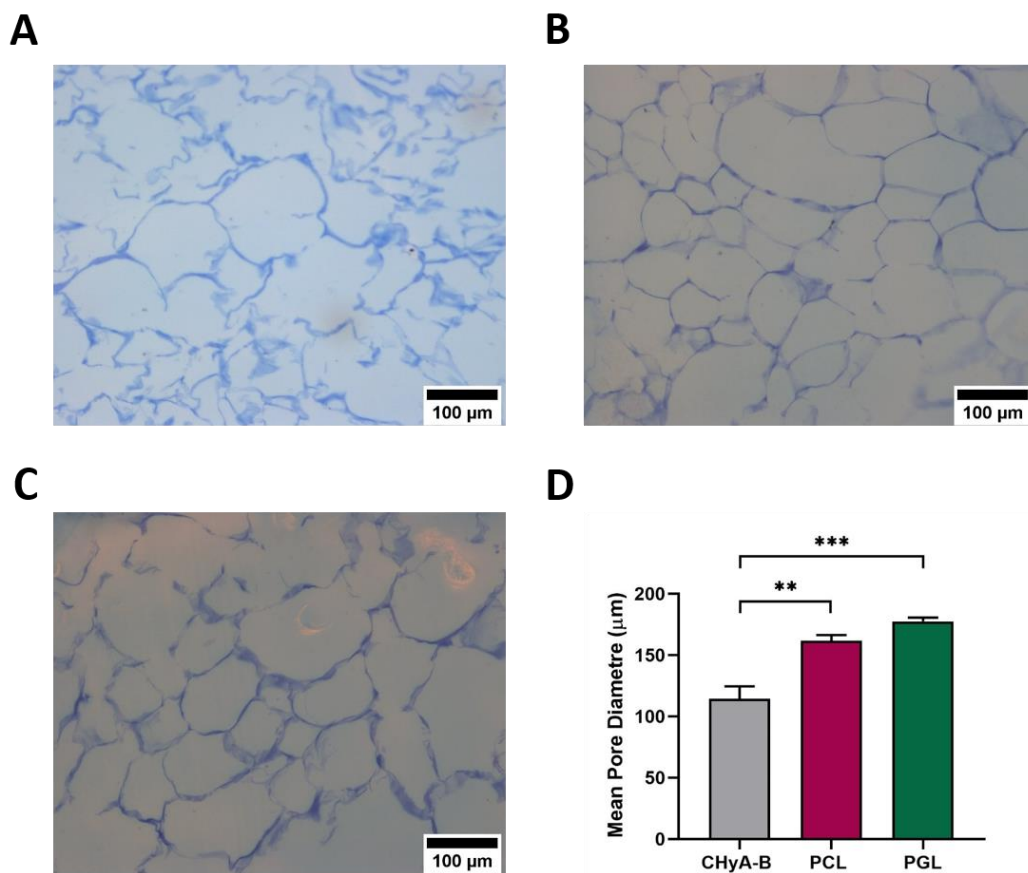
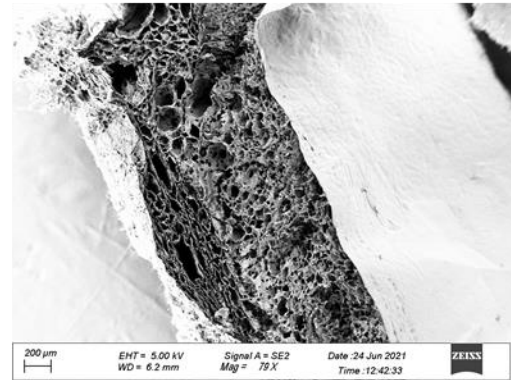
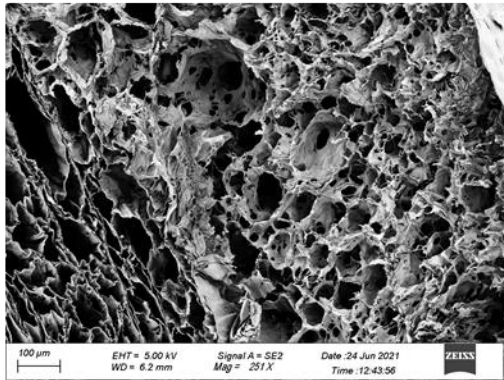
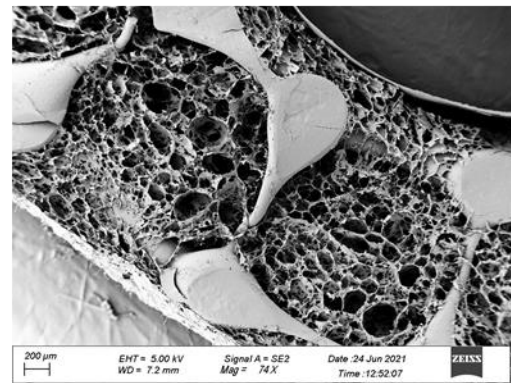
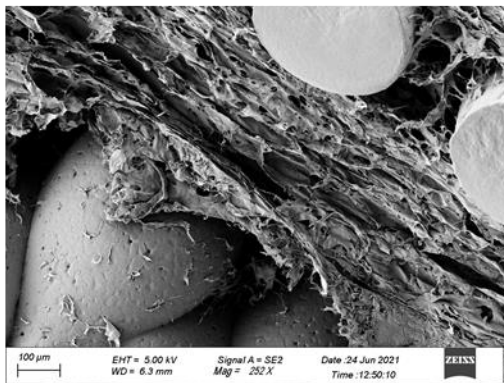


Figure 5. 7. Pore size analysis of tubular tracheal scaffolds. Representative images of toluidine blue staining of (A) CHyA-B, (B) PCL-CHyA-B and (C) PGL-CHyA-B tubular scaffolds. (D) Mean pore diameter of CHyA-B and PCL/PGL-CHyA-B tubular scaffolds. Toluidine blue imaging shows collagen and hyaluronic acid (HyA) stained in dark blue. Results displayed as mean $\pm$ SEM (n=4, **p<0.01, ***p<0.001).

A



B



C

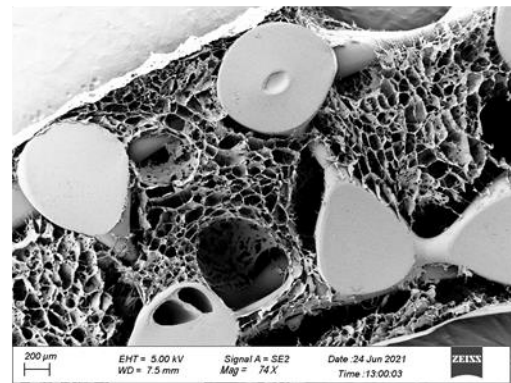
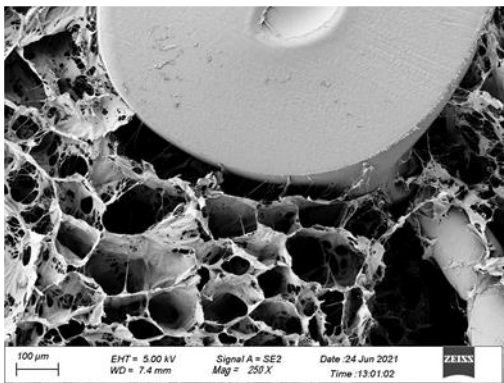


Figure 5. 8. SEM imaging of freeze-dried CHyA-B and PCL- and PGL-CHyA-B tubular scaffolds using a 250X and 77X magnification. Representative images of (A) CHyA-B, (B) PCL- and (C) PGL-CHyA-B tubular scaffolds.

5.3.4 Seeding Conditions Optimisation on PCL-CHyA-B Tubular Scaffolds

Due to the challenges of cell seeding onto tubular constructs, the cell seeding density as well as the seeding time under rotation were first optimised. This was done to achieve successful epithelial attachment within the tubular scaffolds, especially in the CHyA film facing the inner lumen of the scaffolds. Three seeding densities were compared: seeding density A (5×10^5 cells/cm²), B (10×10^5 cells/cm²) and C (20×10^5 cells/cm²) under rotation (0.5 rpm). Seeding density A and B showed higher metabolic activity at day 2 and 7 in comparison to seeding density C (Figure 5.9A). Metabolic activity measured in the construct at day 2 using density C was non-detectable and very significantly decreased when compared to the other densities used, which showed optimal efficiency. Although density A and B proved to be optimal, seeding density A was chosen for further experiments due to a slightly higher metabolic activity measure at day 2, though this was not statistically significant (Figure 5.9A). A range of seeding times under rotation conditions were assessed. A significant increase in day 2 using 4 h under rotation showed a significantly increased metabolic activity in comparison to 1 and 2 h under rotation. No further differences were observed at day 7 (Figure 5.9B). Therefore, it was established that constructs for further experiments will be seeded using 5×10^5 cells/cm² (cell seeding density A) for 4 h at 0.5 rpm rotation to achieve sufficient cellular attachment and viability in the tubular scaffolds.

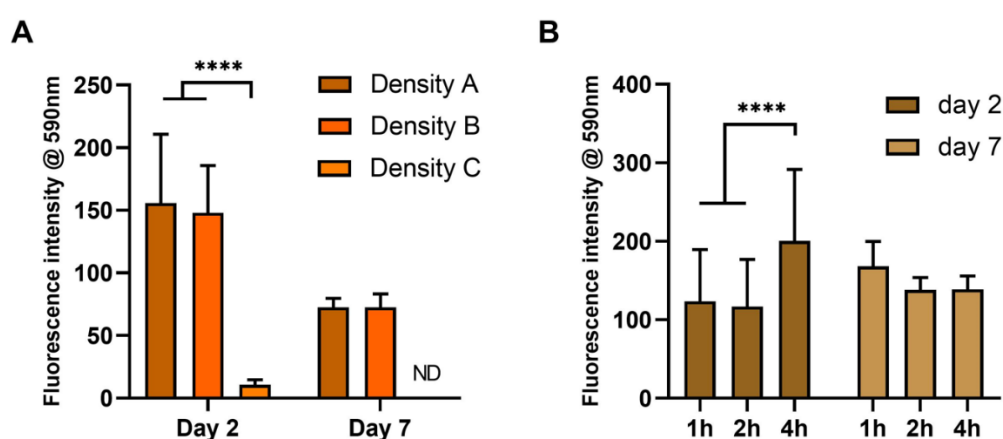


Figure 5. 9. Cellular seeding optimisation on PCL-CHyA-B tubular scaffolds using Calu-3 cells. (A) Optimisation of dynamic cell seeding using three different cell densities, evaluated using AlamarBlue™ at day 2 and day 7. Density A = 5×10^5 cells/cm², Density B = 10×10^5 cells/cm² and Density C = 20×10^5 cells/cm² (n=2). (B) Optimisation of seeding time under rotation (0.5 rpm) using Density A. Results displayed as mean $\pm$ SEM (n=3, ***p<0.001).

5.3.5 Optimisation of Epithelialisation on PCL-CHyA-B Tubular Scaffolds

Once the seeding density and the seeding time were selected, the effect of culture time of Calu-3 epithelial cells on the scaffolds was evaluated to establish the time point at which cell viability and cell coverage of the film in the inner lumen of the scaffold were optimal. Epithelialisation optimisation was only carried out in PCL-CHyA-B tubular scaffolds due to the easy accessibility of PCL, as it is commercially obtained, in comparison to PGL, which needs to be synthesised from its monomer. Metabolic activity from epithelial cells seeded on PCL-CHyA-B tubular scaffolds showed a significant increase at day 7 and 10 compared to day 2 (Figure 5.10A). During the seeding process aimed at epithelial seeding on the CHyA film, Calu-3 cells were also attached to the porous sublayer and therefore metabolic activity evaluated cell activity from the whole tubular scaffold. On the other hand, by removing the CHyA film prior to DNA quantification, a more precise estimation for cellular attachment and growth exclusively on the luminal film could be obtained. DNA content from the cells on the CHyA films also showed a significant increase at day 7 and 10 of culture in comparison to day 2 (Figure 5.10B). Differences between day 7 and 10 were not statistically significant for both metabolic activity and DNA content quantification (Figure 5.10).

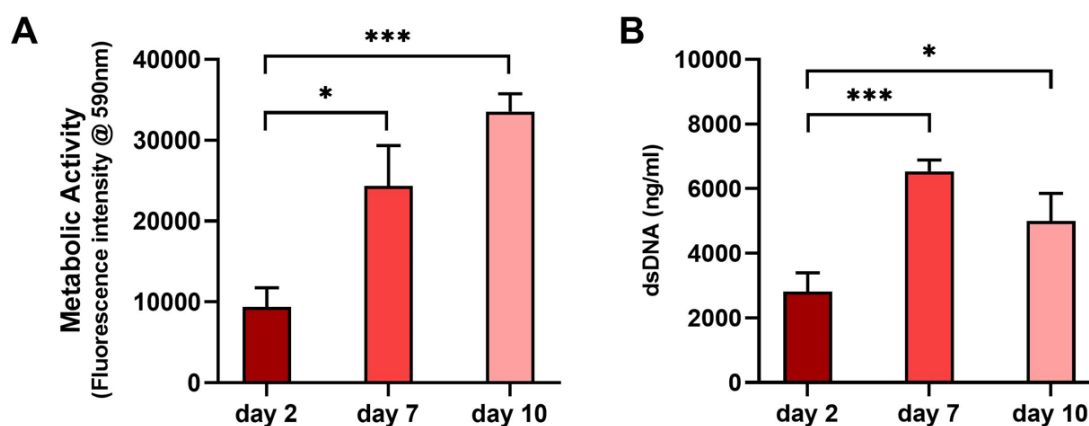


Figure 5. 10. Epithelial cellular growth of Calu-3 cells measured on of PCL-CHyA-B tubular scaffolds seeded with 5×10^5 cells/cm² for 4 h under 0.5 rpm. (A) Cell metabolic activity and (B) DNA content measured from the CHyA film of PCL-CHyA-B scaffolds at day 2, 7 and 10 of culture. Results displayed as mean $\pm$ SEM (n=3, *p<0.05, ***p<0.001).

The coverage of the film on PCL-CHyA-B scaffolds by Calu-3 epithelial cells was confirmed by staining and imaging of the films using phalloidin and DAPI, stains for cellular cytoplasm and nuclei respectively. As show in Figure 5.11A, little cell coverage was seen at day 2, with mostly small clusters of Calu-3 cells found across the CHyA

film. However, by day 7 and 10, an increase in cellular coverage on the film was observed. This was further confirmed by numerically estimating the cellular coverage of the film, with a significant increase at both day 7 and 10 when compared to day 2, while no difference was detected between day 7 and 10 (Figure 5.11B). Although no significant differences were observed between day 7 and 10 for metabolic activity, DNA content and epithelial coverage quantification, day 10 was selected as the time point to assess cellular viability and epithelialisation.

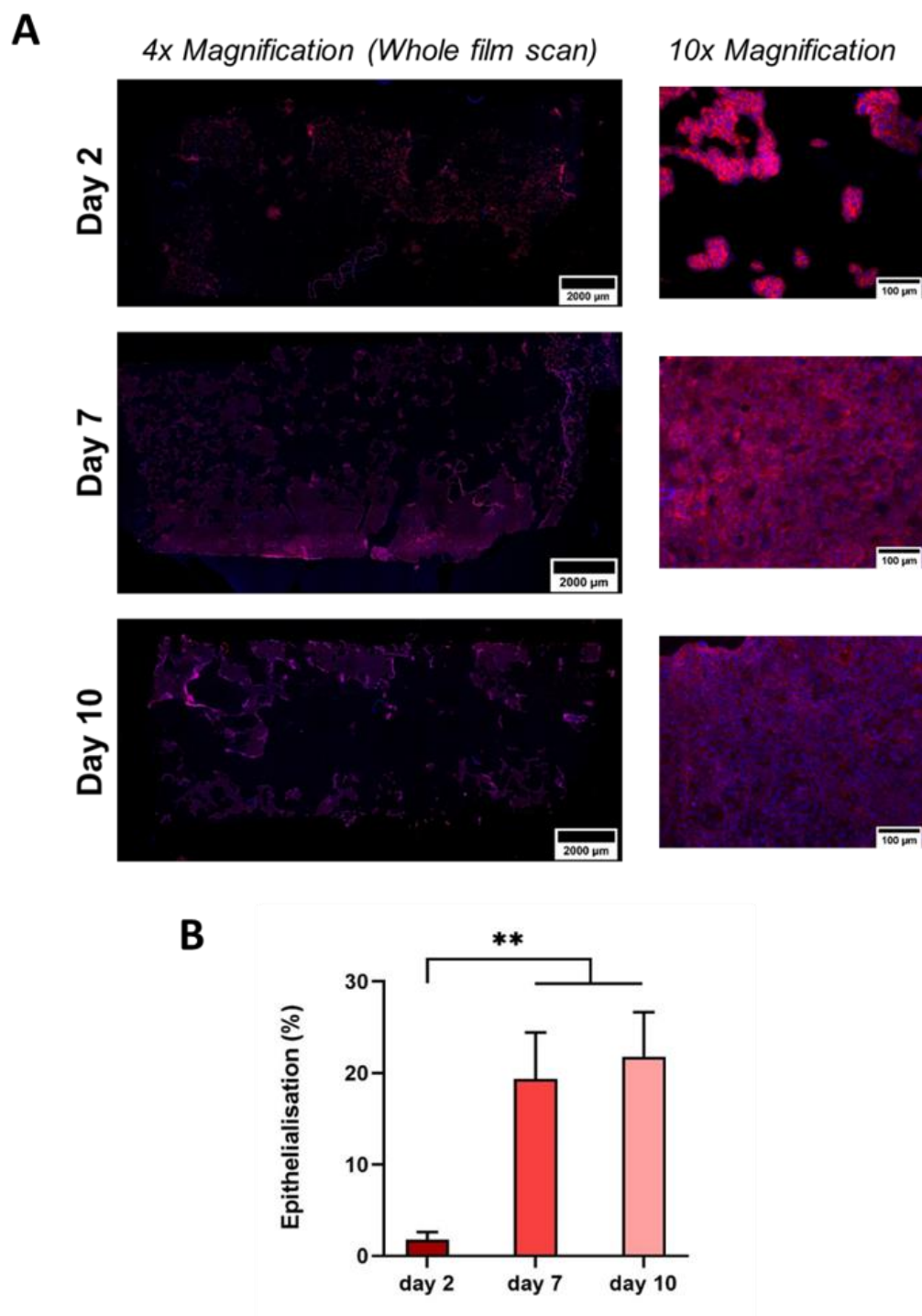


Figure 5. 11. Epithelialisation and cell coverage of the CHyA film of PCL-CHyA-B tubular scaffolds seeded with 5×10^5 cells/cm² Calu-3 cells for 4 h under rotation (0.5 rpm). (A) Representative images of epithelial coverage of the film at day 2, 7 and 10 of culture using DAPI (blue) and phalloidin (red) staining at 4X and 10X magnification. (B) Coverage of the film by epithelial cells (%) at day 2, 7 and 10 of culture. Results displayed as mean $\pm$ SEM (n=3, **p<0.01).

5.3.6 Epithelialisation on PCL- and PGL- CHyA-B Tubular Scaffolds

In order to determine if PGL offered an alternative to PCL as a biomaterial for tracheal tissue engineering, Calu-3 epithelial cells were seeded as above on both PCL- and PGL-CHyA-B tubular scaffolds. Metabolic activity of Calu-3 cells on the scaffolds showed a significant decrease in PGL-CHyA-B compared to PCL-CHyA-B tubular scaffolds at day 10 of culture (Figure 5.12A). This decreased activity may be due to the fact that metabolic activity was measured for the whole scaffold, which includes both the CHyA film as well as the porous collagen sublayer reinforced with the 3D printing polymer design. While metabolic activity performed on the tubular scaffolds accounted for cells seeded on both CHyA film and porous sublayer, DNA quantification could be performed only using the CHyA film to better evaluate seeding differences between PCL and PGL-CHyA-B tubular scaffolds. When only the film was used to evaluate the content of DNA (representative of cell numbers), no significant differences were observed between PCL and PGL-CHyA scaffolds. This demonstrated a similar cellular growth and attachment to the CHyA film without any significant difference in the effect of the polymer used for the reinforcement (Figure 5.12B).

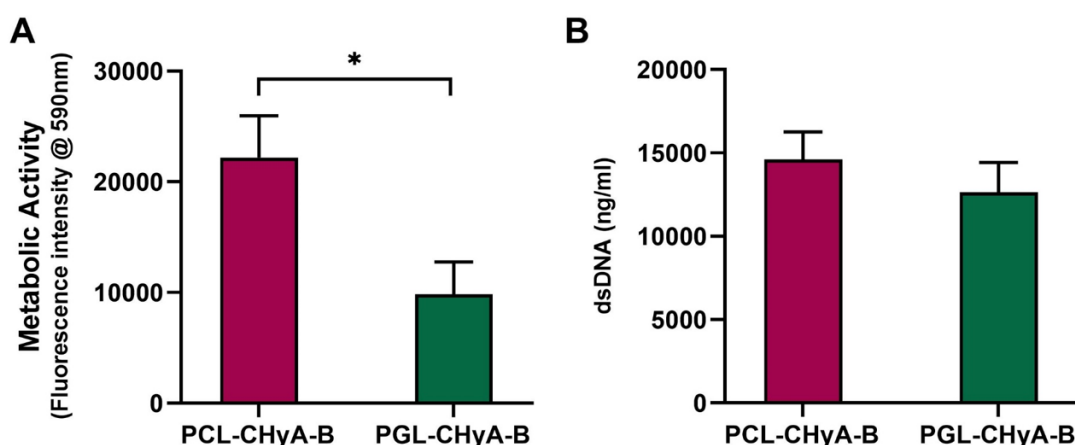


Figure 5. 12. Cellular growth on PCL and PGL-CHyA-B tubular scaffolds. Scaffolds were seeded with 5×10^5 cells/cm² Calu-3 cells for 4 h under rotation (0.5 rpm). (A) Cell metabolic activity from full tubular scaffolds (B) DNA content measured from the CHyA film of PCL and PGL-CHyA-B tubular scaffolds at day 10 of culture. Results displayed as mean $\pm$ SEM (n=3, *p<0.05).

These results were further confirmed by assessing the cell coverage of the CHyA films for each scaffold. There were no significant differences between the epithelialisation of PCL- and PGL-CHyA-B scaffolds with optimal cellular coverage at day 10 of culture

(Figure 5.13). When the total area of the film was evaluated after the phalloidin and DAPI staining, no significant differences were observed between the polymers (Figure 5.13E), with an average 30% coverage on both scaffolds (n=2).

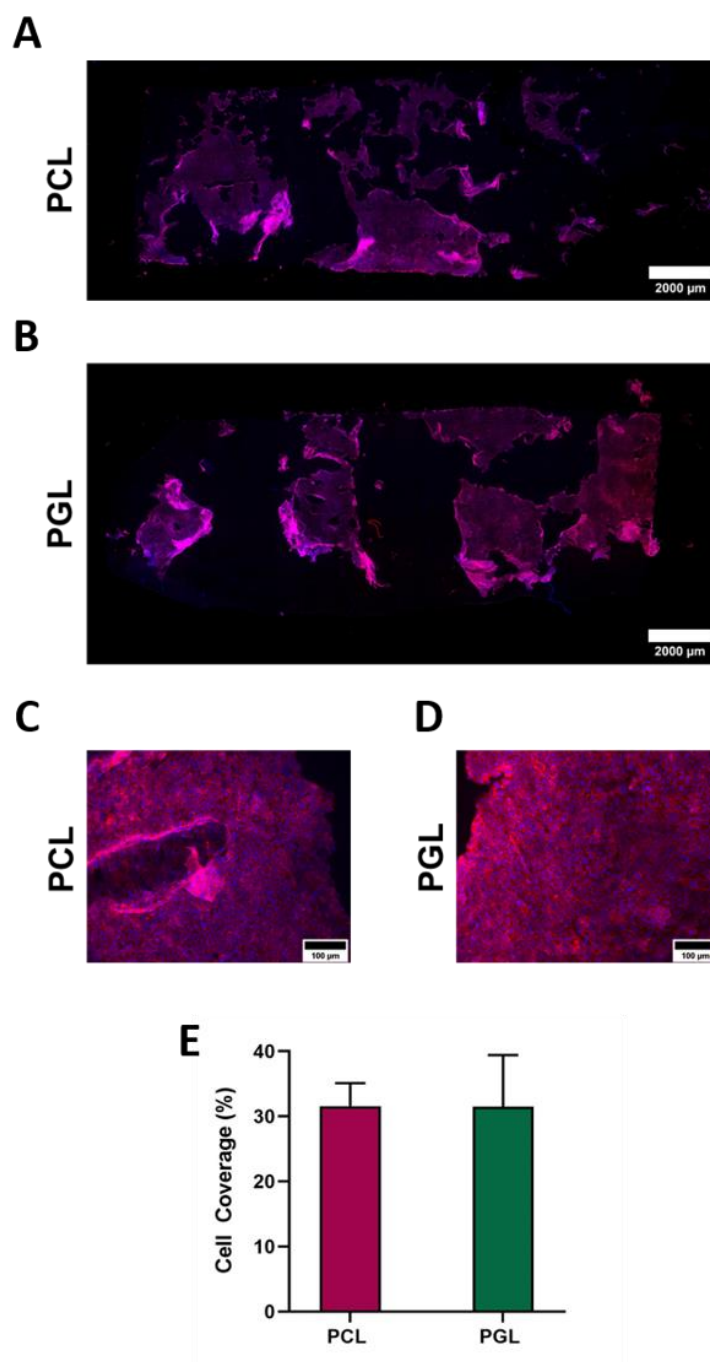


Figure 5. 13. Epithelialisation and cell coverage of the film of PCL- and PGL-CHyA-B tubular scaffolds seeded with 5×10^5 cells/cm² Calu-3 cells for 4 h under rotation (0.5 rpm). Representative images of epithelial coverage of the film from PCL (A) and PGL (B) reinforced CHyA-B tubular scaffolds at day 10 of culture using DAPI (blue) and phalloidin (red) staining at 4X magnification. Representative images of epithelial coverage of the film from PCL- (C) and PGL- CHyA-B tubular scaffolds (D) at 4X magnification. (E) Coverage of the film by epithelial cells (%) at day 10 of culture. Results displayed as mean $\pm$ SEM (n=2, **p<0.01).

5.4 Discussion

Clinical translation of TET approaches has been hampered to-date by poor understanding of the complex needs and requirements of these implants and a lack of effective prototyping⁵³⁹. Increased knowledge in the field and more advanced processing systems such as 3D printing can overcome some of these hurdles. It is now clear that requirements for effective TET include mechanical integrity, sufficient vascularisation and support the formation of respiratory epithelium as a barrier against infection and pathogens⁷². Another key aspect hampering the successful clinical application of these approaches has been the poorly designed clinical development pathways. Taking into account the complexity of restoring whole segmental tracheal defects⁴⁵³, a new approach in the design of studies involving tracheal grafts is needed where firstly the scaffold design and mechanical properties are investigated so that tracheal patency is maintained and stenosis avoided. Secondly, *in vitro* evaluation should be employed to demonstrate the graft's ability to support the formation of epithelial and cartilage tissues and to avoid granulation and inflammation^{105, 540, 541}. Vascularisation studies should also be planned in order to assure the formation of a vasculature network to support neotissue formation^{542, 543}. Once *in vitro* testing shows successful results, *in vivo* implantation in animal models should follow before commencing human trials in order to avoid ethical concerns, scientific misconduct as well as previous medical scandals^{544, 545}. Harnessing 3D printing methods here, it was sought to first develop and design a tubular tracheal scaffold using two polymers of interest in combination with collagen-based scaffolds and demonstrated their mechanical integrity using one well established (PCL) and a novel synthetic polymer (PGL). Once this was achieved, the first steps of an *in vitro* seeding strategy were developed and successfully grew an epithelium on the inner lumen of these scaffolds using Calu-3 cells, showing an estimate epithelial coverage of 30% of the CHyA inner film after 10 days in culture.

We successfully designed and printed both tubular and c-shape scaffold prototypes with both polymers, PCL and PGL, with similar printability. In comparison to approaches used in Chapter 4 to produce lattice structures with PGL, the printing temperature was increased to 100°C to print the tubular and c-shaped constructs while the rest of parameters remained unchanged. This increase in temperature led to better and more accurate prints, comparable to those obtained with PCL (Figure 5.3).

Moreover, PGL (350 mm/min) was printed using a speed higher compared to PCL (260 mm/min), which results in faster prints while precision is maintained, speeding up the development process. This also represents a faster and improved speed compared to other printed materials such as PCL-chitosan composites (60-180 mm/min), PLA (180 mm/min)⁵⁰². Although printing temperature for PGL was increased in comparison to Chapter 4, it still required lower temperatures for printing (100°C) compared to other printable polymers such as polylactic acid (PLA) (220°C) or polyvinyl alcohol (PVA) (180°C), which allows for the use of less complex 3D printers with lower need for higher temperatures⁵⁰². Temperature is a key parameter in 3D printing technology, as it has been demonstrated how changes in temperature can affect the properties of the printed constructs including its printability^{546, 547}. Further analysis on how temperature changes affect the polymer's viscosity, 3D printing process, and the characteristics of the printed polymer should be evaluated to gain a better understanding on the 3D printing behaviour of this novel printable polymer.

One key aspect hampering the development and translation of tracheal substitutes is the lack of a standard mechanical testing protocols and the scarcity of mechanical data from human samples. The complex nature of the trachea results in difficulty when establishing the mechanical requirements needed, which ultimately leads to testing inconsistencies and difficulty in comparing results across literature³⁵. Difficulties in obtaining tracheal samples means that only sections of the organ are tested in many studies, which fails to recapitulate the whole organ mechanical integrity and strength⁵⁴⁸. Moreover, if samples from animal models are used, special attention needs to be paid to the effective translation of this animal data for human prototypes²⁴⁸. Here, we first performed uniaxial compression in both longitudinal and radial directions to evaluate the scaffold behaviour in both testing regimes. As expected, the inclusion of a 3D printed polymer led to increased mechanical integrity across all testing groups compared to CHyA-B scaffolds. Interestingly, PGL constructs exhibited lower Young's modulus when compared to PCL using the same design. This opens the possibility for PGL of being used when slightly lower mechanical integrity is desired (Figure 5.5A). More importantly, both PCL and PGL tubular scaffolds showed stable mechanical properties after continuous cyclic loading while the decrease in the Young's modulus for PGL constructs closely matched previous tracheal scaffolds at an average Young's modulus of 6 MPa⁵⁴⁹. The reported compressive Young's modulus for trachea

constructs, in the range of 1-2 MPa in porcine and rabbit models is lower than the PCL and PGL construct values^{66, 75}. However, it is important to consider differences between human data and animal models, as well as the testing set up used e.g. whole trachea versus tracheal section testing. Moreover, the use of 3D printing allows for rapid design modifications which can easily alter mechanical properties based on needed requirements⁵¹⁵. On the other hand, radial compression of the PCL and PGL tubular constructs showed peak load of 0.7396 and 0.2811 N respectively (Figure 5.5B). This was lower than the compression strength observed in porcine, canine and caprine samples which are between 8-15 N, but closer to those reported for human trachea (0.55-2.32 N)⁵⁵⁰. Finally, there was no significant decrease in the average peak load measured at cycle 1 and 250 for any of the designs tested (Figure 5.6), demonstrating the materials' ability to maintain mechanical integrity over a continuous period of cyclic loading. Comparison of these last mechanical results across literature was not possible, as fatigue testing is not commonly employed in comparison to lateral compression and tensile testing, which are of preferred interest for researchers. Overall, reinforcement of CHyA-B with either PCL or PGL 3D printed constructs significantly increases mechanical properties of the scaffolds for its application in tracheal tissue engineering applications. Comparison to pre-existing data on radial compression for human tracheas and the ability for the 3D printed constructs to withstand repeated cycles of applied force demonstrates that PCL- and PGL-CHyA-B scaffolds could potentially offer a TET approach with the desired mechanical strength. However, further characterisation such as 3 point bending assays and tension testing will be required match properties of 3D printed constructs with those of native tracheas. On the other hand, development of a mechanical testing regime and access to accurate human samples is still needed to boost the development of TETs and its translation into clinical applications.

Building on earlier work in Chapter 4 where CHyA successfully infiltrated the polymer 3D printed construct, we further assessed if this was maintained when using tubular scaffolds. Similar to Chapter 4, excellent CHyA slurry infiltration was observed in PCL and PGL tubular scaffolds as shown by toluidine blue staining and SEM imaging (Figure 5.7A-C, Figure 5.8). The formation of a porous network of collagen and hyaluronic acid within the 3D printed construct would allow for the seeding of the outer portion of the tubular scaffolds with relevant cells e.g. cartilage formation as previously

demonstrated with other scaffolds aiming at tracheal repair^{144, 551}. The presence of the 3D printed tubular scaffolds increased the mean pore diameter of the collagen sublayer from 114.3 μm in CHyA-B tubular scaffolds to 161.8 and 177.6 μm in PCL- and PGL-CHyA scaffolds. The obtained pore sizes are in the range of 100-200 μm , which has been shown to promote proliferation and differentiation of cartilage tissues which would ultimately be expected to grow on the porous outer sublayer of this tubular scaffolds^{552, 553}.

Once adequate scaffold integrity and pore size for cellular attachment and growth was established, a protocol for Calu-3 cell seeding of the tubular scaffolds was established. The final aim of this tubular scaffolds is to support not only the seeding of epithelial cells in the luminal side, but also to support the growth of other tracheal tissue types in the porous collagen sublayer reinforced with 3D printed polymers. Seeding of tubular constructs for tissue engineering applications remains challenging⁵⁵⁴. Several approaches can be found across the literature, including vacuum-centrifugal force-flow based devices, the use of magnetic fields, sedimentary and rotative forces⁵⁵⁴⁻⁵⁵⁶. Here, we developed a simple custom-made device using modified conical tubes with the required space to fit the tubular scaffolds while seeding took place under rotation to support cell distribution across the luminal film layer. Seeding efficiency from this method was adequate when using a seeding density equal to 5×10^5 cells/cm² compared to higher and lower densities, which might be due to a saturation effect and consequent cell death as previously reported (Figure 5.9)^{557, 558}. Seeding of tubular PCL-CHyA-B tubular scaffolds using the optimised seeding density lead to excellent cell growth on the inner CHyA film of the tubular construct as shown by metabolic activity and DNA content measurements (Figure 5.10). This was further confirmed by imaging of the film with epithelial coverage accounting for 20% of the film total area at day 10 of culture. These results demonstrated that cell attachment of Calu-3 epithelial cells to the luminal tubular films was achieved although further modifications would be needed to increase cell growth and epithelial coverage of the film and to avoid unwanted epithelial seeding on the porous sublayer. For example, co-culture seeding of the porous sublayer could be explored as well as increasing time of culture to obtain higher epithelialisation of the luminal film.

PGL tubular scaffolds were developed to serve as an alternative to PCL tubular constructs for tracheal regeneration. PGL is a promising biomaterial for tissue engineering applications, as it is an alternative printable material to PCL and allows for the introduction of post-processing modifications^{170, 173}. PGL-CHyA-B tubular scaffolds consisted of luminal CHyA film seeded with Calu-3 cells and a porous collagen sublayer reinforced with 3D printed PGL. Interestingly, cell metabolic activity was reduced at day 10 of culture when compared to PCL constructs (Figure 5.12A), which can be easily explained due to the cell attachment problems and hydrophobicity observed in Chapter 4. During the rotational cell seeding process, Calu-3 epithelial cells were found to attach to both the inner surface (CHyA film) as well as to the outer porous layer of the construct (CHyA porous sublayer). The CHyA film was removed from the outer porous sublayer reinforced with 3D printed polymer and further processed for measurement of DNA content. No significant differences in DNA content between films from PCL- and PGL-CHyA-B tubular scaffolds were observed showing a similar Calu-3 seeding efficiency on the film for both PCL- and PGL-CHyA-B tubular scaffolds.

The formation of a mature epithelium in tracheal grafts has been proven to be key for the performance of the implant as seen by many attempts using pre-epithelialisation techniques prior to implantation^{189, 314, 539}. Calu-3 cells, epithelial cells of rapid growth displaying key characteristics of the respiratory epithelium, were useful and easy to obtain for the cell numbers optimised in this Chapter for seeding of tubular scaffolds¹⁰⁷. Epithelial coverage of scaffolds for trachea regeneration is rare across literature, therefore hindering comparison with data herein obtained. Epithelial growth on a variety of TET is usually demonstrated via imaging, but no quantification or estimation of the coverage of this scaffolds is performed^{84, 97}. This further hinders clinical translation of TET as full coverage of the implantable scaffold by epithelial cells has not been yet reported. For instance, studies using TET seeded with epithelial cells prior to implantation do not quantify the epithelial coverage and no estimate of a minimum coverage needed for a viable TET can be obtained^{186, 559}. Improvement of epithelial coverage obtained with PCL- and PGL-CHyA-B tubular scaffolds could be addressed by longer culture periods although cell viability should be carefully monitor in 3D or by using co-culture strategies^{346, 560}. Moreover, the epithelium should be matured via introduction of ALI conditions, which will lead to the need of using

specific bioreactors for the culture and maintenance of a mature epithelium. Such specific types of bioreactors have been shortly explored for skin applications and the growth of a respiratory epithelium⁵⁶¹. Cell lines are very useful in early stages of any TE graft development due to its rapid expansion, cost effectiveness and easy accessibility⁵⁶². However, once optimisation and promising results have been obtained using cell lines, primary cells e.g. NHBES should be consider in order to achieve more relevant results and further characterise the scaffold performance⁵⁶³. Apart from assessing the formation of a respiratory epithelium, the porous sublayer of the tubular constructs should subsequently be test to evaluate the formation of other tracheal tissues by using hMSCs, chondrocytes or HUVECs^{499, 564-566}.

5.5 **Conclusion**

Overall, in this Chapter the feasibility of manufacturing 3D printed tubular scaffolds was demonstrated using two different polymers, PCL and PGL as biomaterials to act as a backbone in order to increase mechanical integrity upon incorporation into CHyA-B scaffolds. CHyA-B scaffolds have been shown in Chapter 2 and 3 to be an excellent biomaterial for respiratory tissue engineering applications and these properties have been harnessed here to form a 3D printed regenerative scaffold. Proof of concept work on the seeding of Calu-3 epithelial cells and their support and growth on the scaffolds was carried out for developing promising TE scaffolds aimed at tracheal regeneration. For this, the potential of using PGL, previously optimised as a 3D printing material in Chapter 4, was demonstrated as a novel polymer to reinforce CHyA-B scaffolds and allow for the attachment and growth of epithelial cells in the luminal film of the scaffold. This Chapter led to the following conclusions:

- PCL and PGL constructs can be designed and 3D printed to achieve the desired mechanical properties for a tracheal graft.
- PCL and PGL tubular scaffolds can be combined with CHyA-B scaffolds using a freeze-drying process and retain a collagen pore size suitable for cell attachment and proliferation.
- A protocol for seeding the tubular scaffolds using epithelial cells was developed and enabled the formation of a partial epithelial layer on the luminal film of the scaffold
- PGL shows promise as a novel biomaterial for the manufacture of tissue engineering scaffolds

Thus, at the end of this Chapter it can be conclude 3D printed PCL- and PGL-CHyA tubular scaffolds are as promising prototypes for tracheal repair and regeneration.

Chapter 6

General Discussion and Conclusion

6.1 General Discussion

Respiratory diseases are a leading cause of death and disability worldwide presenting a towering burden on health and society, which includes the five major respiratory diseases: chronic obstructive pulmonary disease (COPD), asthma, pneumonia, lung cancer and tuberculosis⁵. For example, it is estimated that COPD is responsible for the death of approximately 3 million people annually worldwide, affecting a total amount 65 million people⁵⁶⁷. Asthma, an airway disease characterised by the abnormal narrowing, swelling and inflammation of the airways, is believed to have affected 262 million people in 2019⁵⁶⁸. Apart from causing major morbidity and mortality, airway diseases represent an economic burden to society as they are estimated to represent 7.3% of the total health costs in the United States (U.S.) e.g. it is estimated that asthma leads to \$11.3 billion health care costs per year⁵⁶⁹. Although the fight against severe respiratory diseases in recent decades has resulted in positive outcomes with reduced death rates for asthma and COPD patients and a decline in the incidence of lung disorders affecting infants, it is estimated that 41% of deaths in paediatric patients younger than 1 year old can be traced back to respiratory diseases⁵⁶⁹. On the other hand, cutting-edge research into lung diseases, improved epidemiological methodologies and state-of-the-art treatments have led to extraordinary progress in the understanding, prevention and treatment of respiratory diseases^{570, 571}.

Current treatments for airway diseases are predominantly focused on symptomatic relief rather than treating the underlying disease or providing regenerative approaches when required. This can be clearly seen with the turnaround of novel treatments for airways diseases, in which novel therapies are estimated to have a probability as low as 3% for clinical success^{13, 15, 16}. For drugs treating other non-respiratory illnesses, this rate increases to 6-14%^{13, 15}. This can be addressed by better knowledge of airway pathophysiology, increased understanding of airway diseases and better platforms for safety and efficacy testing of pioneering treatments¹⁵. Tissue engineering research could contribute significantly to overcoming some of the current limitations faced in respiratory research²⁴. On one hand, improved tissue-engineered (TE) *in vitro* models of the airway have the potential to provide more accurate three dimensional (3D) representation of lung physiology and disease pathology using human cells compared to the current filter cultures commonly used. They could also reduce the dependence

on pre-clinical animal testing fitting the 3R (Replacement, Reduction and Refinement) principle²²⁷. These TE models also enable the inclusion of human cells allowing testing on human-derived tissues in comparison to other models such as rodent models. Advanced 3D models of the airway that accurately reflect the respiratory epithelium and the translocation of inhaled substances across the epithelial barrier can be harnessed to accelerate and improve the translation of respiratory research by providing platforms to test the safety and efficacy of novel inhaled treatments^{19, 572}. Furthermore, the expertise and technologies evolved in the creation of these 3D airway models can be harnessed for applications in airway tissue regeneration^{573, 574}.

Therefore, the overall aim of this thesis was to develop improved and physiologically-relevant respiratory tissue analogues of the upper airway region that can be used in respiratory drug development and can also be harnessed as tubular constructs for tracheal regeneration. In the first instance, the responses of epithelial cells on a physiologically-relevant bilayered model of the upper airway were compared with conventional filter culture models after apical challenges. A bilayered construct previously developed in our group^{132, 329} was compared for the first time with a standard filter culture model of the airway epithelium. Comparative cell responses were then assessed after apical challenge with a range of materials including substances known for causing inflammatory and toxicological effects on cells: lipopolysaccharide (LPS) and bleomycin. Furthermore, the potential of this model to be employed as a platform for drug screening was investigated. Finally, in the latter portion of this thesis, the bilayered scaffold was further evolved in the creation of tubular constructs for tracheal regeneration.

Here, we sought to apply the previously developed collagen and hyaluronic acid bilayered (CHyA-B) scaffolds for the creation of advanced tissue analogues. CHyA-B scaffolds support the formation of a functional epithelium resembling the *in vivo* tracheobronchial region with key characteristics including barrier integrity, mucus secretion and ciliation¹³². Therefore, in Chapter 2, a 3D TE co-culture model of the tracheobronchial region using CHyA-B scaffolds was manufactured and extensively characterised after exposure to substances known for causing inflammatory and toxicological effects on epithelial cells: LPS and bleomycin^{334, 335, 362, 364, 431}. Lack of consistent and reproducible standards used across *in vitro* models hinders further translation of models of the airways, which makes comparison between them and *in*

vivo physiological responses difficult. In Chapter 2, a systematic and comprehensive study of exposure conditions for LPS and bleomycin was undertaken in the first instance to ensure a robust comparison of the airway epithelial models in the later sections of the work. Optimal exposure conditions for these substances across literature are not consistent, as the different approaches, concentrations and exposure times significantly vary between studies^{335, 431, 575}. The first section of Chapter 2 addressed this lack of consistency and the related work has been already published⁸⁶. Epithelial cells showed higher apical and basolateral secretion of the pro-inflammatory cytokine interleukin-8 (IL-8) after exposure of polarised Calu-3 Transwell® cultures to LPS. Bleomycin exposure resulted in robust toxic effects on the cellular metabolic activity and disruption of the epithelial barrier in polarised Calu-3 cells grown on Transwell® inserts. It is important to note that albeit bleomycin is commonly used for the creation of induced lung injury models^{339, 342}; this is the first report across literature in which bleomycin exposure was systematically optimised on polarised tracheobronchial epithelial cells.

Once the exposure conditions for bacterial and drug-mediated challenges were successfully optimised in conventional two dimensional (2D) models, the responses from Calu-3 epithelial cells grown on CHyA-B scaffolds in co-culture conditions with lung-derived fibroblasts (Wi38 fibroblasts) were compared. Inclusion of an extracellular matrix (ECM)-like environment in a cell culture model provides cell-ECM interactions and the associated mechanical and biomechanical cues to better resemble native tissue architecture^{233, 234, 354}. Moreover, inclusion of multicellular models has been shown to better represent crosstalk between epithelial and other cell types, which allows for better understanding of cellular responses following foreign stimuli and inhaled formulations²²⁸. For example, inclusion of fibroblasts has been shown to reduce airway inflammation by exerting a protective role on epithelial cells³⁸². Different epithelial responses were observed from Calu-3 epithelial cells grown on CHyA-B scaffolds compared to Transwell® cultures, which act as the conventional 2D model used in pharmaceutical research. Firstly, barrier integrity was reduced in CHyA-B scaffolds, which has already been reported in other 3D airway and intestinal mucosal models^{106, 359} and denotes the formation of weaker tight junctions although barrier formation remains unaffected³⁶¹. By seeding intestinal cell lines (Caco-2 cells) on 3D collagen hydrogels, Macedo et al. were able to demonstrate reduced transepithelial

electrical resistance (TEER) values when cells were grown on 3D compared to standard 2D models, which better reflects *in vivo* values of the intestinal epithelium. Introduction of an ECM-like environment resulted in the formation of weaker tight junctions compared to 2D models, which tend to show higher TEER values mainly due to overexpression of claudins, which act as major determinants of barrier function³⁶¹. Different toxicological responses were also seen in CHyA-B epithelial co-cultures, with higher toxicity versus epithelial cultures on Transwell® inserts after exposure to bleomycin. Increased cell sensitivity in complex 3D multicellular models has already been described and is hypothesised to better mimic *in vivo* responses^{31, 368}. Due to its scarcity, it is challenging to draw similarities with actual human data. However, it is believed that higher sensitivity in 3D models is due to reduced nutrient supply compared to 2D cell cultures and higher basal oxidative stress, which might better represent *in vivo* conditions^{31, 369}. Differential inflammatory responses were also detected, with increased basolateral cytokine secretion from CHyA-B cultures compared to the conventional epithelial models used. Increased levels of IL-8 were detected from Calu-3 cells grown on CHyA-B scaffolds after introduction of co-culture strategies with lung-fibroblasts. Culturing several cell types such as fibroblasts, colon organoid cells and endothelial cells in a 3D environment has been reported to significantly increase cytokine secretion, which is believed to drive recruitment of immune cells following epithelial damage better resembling *in vivo* responses^{374-377, 382}.

It can be concluded that CHyA-B co-cultures of epithelial cells and lung fibroblast represent an improved *in vitro* model of the airways in which better modelling of the ECM-like environment as well as crosstalk between cell types provides more accurate responses to the inhalation of irritant substances. For instance, *in vivo* injection of 1 µg *Pseudomonas aeruginosa* (*P.aeruginosa*)-derived LPS in rats resulted in increased inflammation showing a six-fold increase in the numbers of leukocytes and significant increases of macrophages, neutrophil and lymphocyte counts 4 h after exposure⁵⁷⁶. Moreover, lipid peroxidation products that act as marker for inflammation were increased in mice 24 h after LPS exposure using a concentration of 5 mg/kg mice body weight⁵⁷⁷. This correlates with findings in the present work where increased IL-8 and interleukin (IL-6) is expressed by Calu-3 cells cultured on CHyA-B scaffolds. Furthermore, higher secretion of IL-8 and IL-6 following LPS treatment has already

been reported using advanced immunocompetent cell models of the airway consisting of human lung epithelial cells and macrophages⁵⁷⁶. Epithelial cell responses from Calu-3 cells grown on CHyA-B scaffolds after exposure to bleomycin are similar to the *in vivo* inflammation and apoptosis observed in bleomycin-induced lung injury models 7 days after administration³³⁹. This has also been reported using rodent models showing an increased inflammation 7 days after injection of 4 mg/kg bleomycin⁵⁷⁸. The toxicological and pro-inflammatory responses of the cells observed in our 3D models were significantly different to standard filter cultures of the same cells and similar to *in vivo* studies, highlighting the importance of modelling the ECM and multicellular environment. The use of TE airway models has the potential to increase the understanding of epithelial responses by better mimicking *in vivo* exposure, and could act as screening tools for drug development.

Respiratory medicine suffers from disappointing clinical translation of novel therapies, for which better tools to screen nanomaterials and nanomedicines are desperately needed⁵⁷⁹. Three materials were selected as test nanoparticles: two well-known materials previously assessed in epithelial cultures; poly(lactic-co-glycolic acid) (PLGA) nanoparticles and carbon nanotubes (CNTs) alongside with novel nanomaterials being developed for respiratory applications^{154, 402, 403, 407}. Short-term (at day 15 of culture, 24 h after exposure) responses of CHyA-B Calu-3 epithelial co-cultures to PLGA and CNT exposure were similar to those of previous studies. Longer-term (day 23 of culture, 9 days after exposure) epithelial responses after exposure to PLGA and CNTs also matched those previously reported for similar concentrations⁴³⁹⁻⁴⁴¹. Literature findings show that PLGA does not cause short-term inflammation or toxicity upon exposure in A549 and BEAS-2B airway cells⁴¹³. On the other hand, studies focusing on CNTs showed increased inflammation in lung micro tissue models while CNTs similar in size to the ones used in this work did not led to increased toxicity^{436 253}. Bioinspired star-shaped nanoparticles (G5 nanoparticles) have been developed in-house in RCSI University of Medicine and Health Sciences for applications in drug delivery including for bone tissue engineering and in cystic fibrosis research^{402-404, 416}. Short-term responses of CHyA-B epithelial co-cultures to G5 nanoparticle exposure showed an increase in toxicity and inflammation compared to responses seen in the 2D models. This provides an opportunity for this platform to offer offering greater sensitivity for early detection of inflammatory responses.

However, this was performed using single dosage exposure and therefore, further dose-response studies would be required to better interrogate the effect of this novel nanoparticles in epithelial cells. This finding demonstrates how 3D *in vitro* models can detect adverse effects that might go unnoticed using common 2D models. After CHyA-B co-culture exposure to G5 miR nanoparticles, a significant increase of IL-8 and IL-6 in CHyA-B co-cultures versus Transwell® monocultures was observed long-term, which demonstrated the feasibility of assessing exposure-induced inflammation using this 3D co-culture model. This type of 3D model presents an innovative tool for pre-screening nanomedicines prior to selection for *in vivo* testing. 3D ECM-like scaffold co-cultures of the airways would appear to offer greater sensitivity to challenges compared to the conventional filter culture models of the airway. An increase sensitivity of 3D models has been already reported, in which 3D organoid models of CAL27 cells, an oral squamous cell carcinoma cell line, showed increased sensitivity to applied treatments³¹. This was also detected using Caco-2 3D cultures showing higher sensitivity to ZnO nanoparticles compared to 2D cultures³⁶⁹. Therefore, it can be concluded that CHyA-B scaffold co-cultures of airway cells offer a potentially innovative approach to assessing nanomaterials and nanomedicines *in vitro*.

CHyA-B scaffolds have been presented here and in previous work as a platform for supporting airway cell growth and differentiation^{86, 132, 329}. We have demonstrated that this bilayered construct can be used to support the formation and differentiation of a respiratory epithelium. It can be further combined with small molecules e.g. retinoic acid to develop bioactive materials to enhance epithelialisation for pioneering strategies in tracheal replacement^{25, 132}. Based on the extensive knowledge acquired with CHyA-B scaffolds, it was hypothesised that they could be used to solve the need for new materials and approaches for tracheal regeneration and repair. Current tissue-engineered trachea (TET) approaches often do not succeed in clinical translation due to weak mechanical integrity and poor regeneration of airway tissues⁷². Therefore, the next step was to evaluate the potential of CHyA-B scaffolds as TET grafts. Any TE graft aimed at tracheal regeneration needs to have the requisite mechanical properties within a tubular construct to maintain airway patency and avoid stenosis, one of the main reasons hindering further translation of these approaches^{72, 255, 296, 539}. As seen from previous studies, stenosis and airway patency have proved challenging in previous attempts^{35, 72, 158, 309, 539}. While the CHyA-B scaffolds have the appropriate

composition, ultrastructure and porosity to support airway cell growth, as a standalone material collagen and hyaluronic acid (CHyA) would not provide the mechanical strength required for a tubular TET¹³². Consequently, these natural materials require a tubular backbone to provide structural support and mechanical integrity. 3D printing has emerged as a promising process for TET manufacture with the potential to ultimately manufacture personalised tracheal implants^{179, 211, 219, 220, 505}. Research of the literature highlighted the feasibility of 3D printing for the development of strong tracheal constructs, 3D printing was selected as a manufacturing technique for reinforcement of CHyA-B scaffolds^{209, 211, 219-221}. Therefore, the overall goal was to 3D print a tubular backbone using synthetic materials that could be used to support the CHyA-B scaffold.

3D printing is based on the extrusion of polymers through fine nozzles that allow for controlled deposition of polymer fibres that can be easily designed into desired geometries^{179, 505}. Current synthetic printable materials including PLGA, poly(lactic acid) (PLA) and poly(etheretherketone) (PEEK) show important limitations: high printing temperatures and raw material's high price^{129, 514, 580, 581}. Polycaprolactone (PCL) is a 3D printable synthetic biomaterial that has previously been used for the development of TE scaffolds and it is a U.S. Food and Drug Administration (FDA) approved biomaterial¹⁶². It has been incorporated into several tracheal TE scaffolds but it suffers from reduced cell attachment and growth when used as a standalone material^{156, 219, 221}. To overcome this, synthetic polymers such as PCL are usually employed in combination with natural polymers to reduce scaffold's hydrophobicity and improve cell attachment^{144, 221, 504, 581}. The development of novel materials that can be easily processed, provide mechanical integrity to the graft and support cell growth and attachment has the potential to be a great innovation for the TET field. The printability of a novel biomaterial, polyglycolide (PGL), was investigated. PGL is a biocompatible polymer previously used in the development of TE scaffolds using electrospinning and film-based technologies, but never previously processed by 3D printing^{170, 173, 485}. PGL has a low melting point allowing the use of simple printers such as the ones employed with PCL, but its unique chemistry allows for easy post-printing modifications¹⁷⁰. While conventional polymers such as PCL are difficult to modify to increase its cellular performance, the chemistry of the PGL scaffolds enables surface

modifications such as immobilisation of Arg-Gly-Asp (RGD) sequences to increase cell attachment¹⁷⁰.

In Chapter 4 the printability of PGL and its biocompatibility for tissue engineering applications was investigated. As noted above, this was the first attempt in literature to 3D print PGL, which showed excellent printability comparable to PCL. PGL's printability was investigated using the lattice geometry, consisting of 8 printed layers 2 mm in height and a width of 7.5 mm. PGL was successfully printed using a temperature of 70°C and a pressure of 55 PSI, both reduced compared to the parameters used for PCL 37kDa (100°C, 90 PSI) and PCL 50kDa (100°C, 80 PSI). This temperature is also inferior to the one used for other printable polymers such as PLGA, PEEK, poly(vinyl alcohol) (PVA) and polybutylene terephthalate (PBT), over 200°C. This resulted in a faster printing speed of PGL (350 mm/min) compared to 280 mm/min and 80 mm/min used for PCL 37 kDa and PCL 50 kDa respectively. For further studies on biocompatibility, the 3D printed geometry was used and 3D printed PGL discs were then combined with CHyA porous scaffolds as a proof of concept for the development of tubular scaffolds later in this thesis. PCL-CHyA and PGL-CHyA scaffolds were then seeded using human mesenchymal stem cells (hMSCs), cells commonly used in biocompatibility testing of biomaterials^{582, 583}. PGL-CHyA disc scaffolds showed low cellular attachment compared to PCL-37-CHyA and PCL-50-CHyA discs. However, biocompatibility was further demonstrated with minimal toxic effects of the polymer and cellular presence in the scaffolds up to 14 days in culture. It was hypothesised that the increased hydrophobicity of PGL versus PCL could have played a role in the lower cellular attachment observed. Interestingly, PGL's hydrophobicity has already been demonstrated to be tunable upon introduction of decorative post-processing modifications using poly(acrylic acid) (PAA) and amino groups^{170, 506}. A change in hydrophobicity was observed upon introduction of these modifications, which could help increase cellular attachment to PGL scaffolds. Moreover, a higher crystallinity observed for PGL in comparison to PCL might also explain the reduced cellular attachment as previously reported. It is believed that a higher crystallinity results in reduced gas diffusivity impacting the biocompatibility of the polymer used⁵²³. In Chapter 4, we demonstrated the feasibility of using PGL as a printable material. While PGL appeared non-toxic for those cells attaching to the polymer's surface as previously shown when using PGL electrospun scaffolds^{170, 173},

⁴⁸⁵, it was clear that further work on introducing surface modifications was needed to increase initial cellular attachment and growth. It is also important to discuss a drawback of using PGL in comparison to other polymers e.g. PCL. Currently, PGL cannot be obtained commercially and needs to be synthesised from its monomer, which might not always be easily accessible for research purposes and therefore work on establishing a robust supply chain is needed to further proceed with the use of PGL in TE applications. Moreover, its synthesis is currently restricted to chemistry applications with manufactured quantities being low –around 15 g per synthesis– and will require scaling-up to meet the requirements needed for widespread 3D printing usage.

The development of TET scaffolds for extensive tracheal defects still remains an unmet clinical need due to the lack of robust approaches that successfully addresses all TET requirements: mechanical integrity, formation of a respiratory epithelium and vascularisation to support the formation of neotissues around the implant^{189, 206, 267}. Moreover, there is a lack of extensive *in vitro* characterisation of TET grafts prior to implantation, resulting in mismatched properties and poor success after *in vivo* implantation⁵³⁹. Several examples can be found across the literature focusing on the use of 3D printed TET scaffolds. *In vitro* characterisation studies have been carried out showing the promise of this technique^{175, 220, 221, 584} while *in vivo* implantation has been performed in different animal models and has helped in highlighting the essential requirements for tracheal grafts^{217, 219, 220}. Finally, examples in paediatric patients and adults suffering from tracheobronchomalacia (TBM) have been carried out with successful outcomes^{211, 214, 315, 316}. However, translation of TET grafts remains challenging with stenosis, tissue granulation, inflammation and airway collapse being observed during *in vivo* studies^{188, 215, 219, 453, 584}. In Chapter 5, an innovative approach to manufacture TET scaffolds was developed by combining the knowledge from previous Chapters to create a 3D printed polymeric tubular backbone coated with the CHyA-B scaffolds using a freeze-drying process^{86, 132}. Here, it was sought to develop tubular tracheal scaffolds that could help to overcome the two first limitations of TET grafts: adequate mechanical strength while allowing for the formation of a respiratory epithelium in the inner lumen of the TET graft. PCL and PGL were both successfully printed into tubular designs that were combined with CHyA-B scaffolds to develop scaffolds with adequate mechanical properties and porosities for tracheal

replacement. The potential of these scaffolds to support the attachment and growth of epithelial cells with the final goal of forming a respiratory epithelium in the inner CHyA film of the scaffolds was then assessed. The formation of a respiratory epithelium in TET scaffolds is key to overcoming inflammation and infection after pathogen inhalation¹⁸⁹. After developing a seeding protocol using Calu-3 cells on PCL-CHyA-B scaffolds, PGL-CHyA-B scaffolds were also seeded with Calu-3 cells on the inner lumen of the scaffold. Calu-3 cells were able to attach to the inner film and were able to grow and populate the film over the culture period with similar results observed between PCL- and PGL-CHyA-B scaffolds. Epithelial growth on TET grafts is usually demonstrated through imaging techniques with no quantification of epithelial coverage reported across literature^{84, 97}. Therefore, data obtained here is difficult to compare with other approaches investigated, which further hinders the translation of this TET grafts as a required epithelialisation values needed for a successful implantation has yet to be reported. In Chapter 5, we further confirm the printability and versatility of PGL that could be employed as a novel material for the development of TET grafts with mechanical integrity and support for epithelialisation. PGL stands as a promising potential material for TE applications, but it is apparent from this work that surface modifications on the polymer will be critical to increase cell attachment. This advantage could allow for the use of PGL as a standalone material without the need for combination with natural polymers as an effective tool for tracheal repair and other TE applications.

6.2 Future Work

Results presented have highlighted the potential for carefully designed TE approaches to (i) support drug development and (ii) to enable tracheal regeneration. The results presented throughout this thesis have provided evidence for the potential of CHyA-B scaffolds in the development of advanced *in vitro* models of the airway for drug formulation and testing applications, but can also be employed for approaches aimed at developing TET grafts for extensive tracheal defects. In order to fully realise this potential, additional work is required as outlined below.

During the development of the *in vitro* airway model in Chapter 2 & 3, epithelial cells were co-cultured with lung-derived fibroblast. There is still space to further increase the complexity of this model to better represent the interactions of epithelial cells with immune cells such as macrophages and dendritic cells. For instance, several 3D airway models have already included such cell types e.g. lung and bronchial fibroblasts, endothelial cells, neutrophils and macrophages to better understand the crosstalk between the epithelium and the underlying cell populations^{232, 370-372, 585}. The inclusion of relevant immune cells more closely mimics the immunological responses of the tracheobronchial epithelium to foreign substances. It has been demonstrated that after exposure of epithelial cells to enzymatically active allergens, recovery was faster in the presence of co-culture conditions while dendritic cell migration in the 3D scaffold was enhanced and allows for cell migration after exposure¹⁰⁶. Challenges (LPS and bleomycin) and nanomaterials/nanomedicines were added directly to the apical side of the ALI cultures under submerged conditions. To better mimic inhaled exposure when screening for drugs or drug formulations, alternative inhalation exposure methods could be used such as nebulizers, gas generators, microsprayers or commercially available exposure chambers¹⁹. Integration of the 3D CHyA-B scaffold cultures with an aerosol exposure system would be an interesting and useful next step.

There is a growing interest in the field of nanomedicines but there is a need to better understand the biological and nanomaterial interface, and these 3D models could provide useful tools for some of this work. For example, it is important to understand the effect of inhaled nanomaterials and/or nanomedicines and closely monitor any possible changes on cilia, another key characteristic of the airway epithelium. Research focusing on the ciliation of the respiratory epithelium is of crucial interest in

airway diseases as cilia dysfunction critically endangers the mucociliary clearance in several lung diseases⁵⁸⁶. Evaluation of changes of the mucus layer on top of the epithelial co-cultures can also be of great interest for assessing nanoparticle adherence and translocation. For instance, it has been demonstrated that nanoparticles avoiding adherence to the mucus layer provide a longer lasting and more uniform drug delivery⁵⁸⁷, while nanoparticles altering mucin expression can be of interest in the treatment of cystic fibrosis⁵⁸⁸. Therefore, it is important to be able to study the interaction of nanoparticles and/or nanomedicines with the mucus layer of the airway. The model herein, produces a mucus layer on top of the epithelial monolayer, could be used as platform to study such interactions¹³². In Chapter 3 only three nanoparticles were evaluated using the 3D CHyA-B co-culture model; PLGA nanoparticles, CNTs and G5 nanoparticles. However, it is important to note that a wider range of nanomaterials was intended to be screened including PLA or polyethylene glycol (PEG), but reduction in lab access and supply from COVID-19 impacted this Chapter. Moreover, inclusion of the same material-based nanoparticles of different sizes and surface charge would have been carried out to test whether the model could identify changes in the epithelial responses to different nanoparticles. Finally, G5 nanoparticles were tested on the models using a single dose (G5 miR) previously optimised within our group⁴¹⁶ or a negative control (G5 NC). The original experimental work plan included a dose-response study of these novel nanomedicines on the model to further optimise the therapeutic dose as well as detect any adverse effects related to the doses used.

In Chapter 4 and 5 we demonstrated the printability of the novel biomaterial, PGL, and how 3D printed scaffolds made with this synthetic polymer can be applied in tissue engineering applications. However, PGL scaffolds suffered from low initial cellular attachment when seeded with hMSCs that hindered further cellular growth on the scaffolds. Previously, it has been hypothesised that this behavior could be related to the hydrophobicity of the polymer. To overcome it, polymer blends could be investigated such as the development of PCL/PGL scaffolds as previously reported with other materials^{221, 466, 467, 503}. For example, blends of PCL/nano-hydroxyapatite (PCL/nHA) for bone tissue engineering using melt-blended, powder-blended and solid solvent techniques have been demonstrated to maintain printability properties and resulted in 3D scaffolds with high degree of micro-porosity⁵⁸⁹. PCL has also been 3D

printed in combination with PEG to develop scaffolds with increased hydrophilicity resulting in enhanced scaffold's wettability leading to increased cellular attachment and proliferation⁵⁹⁰. Chemistry approaches could also be investigated to overcome poor cellular attachment as PGL scaffolds can be effectively functionalised reducing the system's hydrophobicity and increasing cellular growth. For example, Ates et al. reported the functionalisation of PGL films by combining enzymatic ring opening and thiol-ene coupling reactions⁵⁰⁶. A change in the film's hydrophilicity was observed after decorating the films with PAA. More recently, electrospun PGL scaffolds were cross-linked for its application in drug delivery applications but showed a lack of recognition sites to support cell adhesion and proliferation¹⁷³. De Oliveira et al. addressed this problem by functionalizing PGL fibres with amine groups resulting in hydrophilic fibres. These functionalised scaffolds were further functionalised with RGD sequences and were seeded with hMSCs. It was demonstrated that the post-processing functionalisation led to higher DNA content and increased cellular proliferation in RGD-functionalised scaffolds compared to non-functionalised scaffolds¹⁷⁰. The process developed for introducing RGD-modifications on the electrospun PGL scaffolds involved the use of solvents, which would not be compatible with the 3D printing process for PGL. Therefore, the first objective should be the development of a process that allows cross-linking of 3D printed scaffolds, for example a strategy that allows the combination of PGL with photoinitiators before polymer melting during 3D printing⁵⁹¹. Alternatively, post-printing cross-linking could be performed to allow further functionalisation, for example, 3D photopolymerisation could be investigated. Here, a mixture of PGL and a relevant photoinitiator could be 3D printed while UV is applied to the newly deposited fibres to achieve polymer cross-linking. However, the low accessible layer thickness for cross-linking (around 100 µm), slow printing rates and the side reactions due to prolonged exposure to UV represent limitations for this technique⁵⁹¹. Once cross-linking of 3D printed scaffolds is achieved, functionalisation using amino groups and RGD sequences should be further tailored to be compatible with the 3D printing process. The development of PGL 3D printed scaffolds could represent a step towards developing more functional biomaterials easily processed and adapted to match patient's specific requirements using CT imaging and advanced computer-modelling techniques.

It has become apparent that the mechanical properties of TET grafts are critical to maintain airway patency but there is a lack of standard protocols available for effective prototype characterisation^{22, 72}. Therefore, there is a need for better data from either human samples or animal models, taking into account the differences with human anatomy if using the last one. Standards protocols for the evaluation of the desired mechanical properties a graft must possess should be develop and should include a wide range of mechanical test to deeply characterise the scaffolds (e.g. compression, tensile testing, 3 point bending and fatigue testing). This will help researchers in better designing scaffolds with the desired properties avoiding airway collapse and stenosis induced via poor inefficient designs.

Here the epithelialisation of the CHyA film of the constructs was demonstrated. To achieve greater epithelialisation co-culture strategies or longer culture periods could be investigated. Combination of co-culture strategies of epithelial cells with relevant cell types in the porous sublayer reinforced with the polymer backbone such as fibroblasts or mesenchymal stem cells (MSCs) could enhance the epithelialisation and coverage of the CHyA films. For example, co-culture with fibroblast and endothelial cells has been shown to enhance barrier properties and could be harnessed to increase epithelial growth^{205, 592}. Zhang et al. demonstrated how seeding of tracheal basal cells favoured rapid epithelialisation of TET upon *in vivo* implantation. Moreover, they were also able to increase epithelial proliferation by *in vitro* co-culturing with embryonic fibroblast cells⁵⁹³. Another alternative to increase epithelialisation *in vitro* would be the use of bioreactors, as effective use of bioreactors has already been reported to increase cell proliferation of chondrocytes seeded on PCL/collagen TET scaffolds³⁰². The use of rotating bioreactors has also been found to be useful in supporting the growth of NHBES cells on porous collagen/PTFE membranes showing improved respiratory epithelium characteristics compared to static cultures⁵⁶¹. Supplementing cell culture media with growth factors crucial for airway growth and differentiation such as fibroblast growth factors (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) could help boost cell growth on the scaffold⁵⁹⁴.

Many studies demonstrate the formation of one important tissue type on tracheal TE scaffolds e.g. epithelium and cartilage followed by immediate *in vivo* evaluation. Many of these candidates failed *in vivo* with evidence of local inflammation and granulation

which flags that focusing on a single tracheal tissue type was not adequate to support full regeneration. Native tracheal tissue is very complex, and for a successful TET graft all tracheal tissues must be supported by the graft and its formation firstly demonstrated *in vitro*⁷³. Therefore, future work would include the use of primary chondrocytes or differentiating hMSCs towards the chondrogenic lineage within the scaffold^{564, 566}. The vascularisation could be enhanced by co-culture of human umbilical vein endothelial cells (HUVECS) as previously reported^{543, 595}. Nonetheless, further pre-clinical development would be needed to fully characterise *in vitro* the potential of the scaffold to regenerate tracheal tissues followed by *in vivo* implantation in relevant models to assure mechanical integrity. Moreover, *ex vivo* lung perfusion (EVLP) models could be employed to investigate the air-tightness of epithelialised constructs and their ability to maintain airway patency prior to *in vivo* assessment⁵⁹⁶.

6.3 Concluding Remarks

The research presented in this thesis has focused on the use of biomaterials for the development of 3D models of the airway and prototypes for tracheal replacement and regeneration. We demonstrated how epithelial responses from the 3D CHyA-B co-culture models are different from commonly used airway models, resembling more closely those observed in the *in vivo* tracheobronchial epithelium. We further harnessed this model for testing of nanoparticles and nanomedicines used in drug delivery applications to act as a 3D platform for safety and toxicity testing. Moreover, the combination of CHyA-B scaffolds with 3D printed synthetic polymer backbones was evaluated for tracheal tissue engineering applications. We explored appropriate synthetic materials for the tubular backbone manufacture including PCL and a novel biomaterial PGL. Its suitability for 3D printing was demonstrated here for the first time. The integration of the CHyA-B scaffolds with 3D Printed tubular PCL and PGL constructs produced scaffolds with the potential to be used in tracheal tissue engineering. Preliminary mechanical testing, porosity assessment and cell studies demonstrating epithelialisation on these prototypes outline their promise as TET approaches for tracheal regeneration and repair.

Bibliography

1. Borg, S.; Townsend, J., The economic burden of lung disease European Lung White Book: Respiratory Health and Disease in Europe. *Sheffield, European Respiratory Society* **2013**, 16-27.
2. Marciniuk, D. D.; Schraufnagel, D. E., *The global impact of respiratory disease*. European Respiratory Society: 2017.
3. Williams, S.; Sheikh, A.; Campbell, H.; Fitch, N.; Griffiths, C.; Heyderman, R. S.; Jordan, R. E.; Katikireddi, S. V.; Tsiligianni, I.; Obasi, A., Respiratory research funding is inadequate, inequitable, and a missed opportunity. *The Lancet Respiratory Medicine* **2020**, 8 (8), e67-e68.
4. Lacroix, G.; Koch, W.; Ritter, D.; Gutleb, A. C.; Larsen, S. T.; Loret, T.; Zanetti, F.; Constant, S.; Chortarea, S.; Rothen-Rutishauser, B.; Hiemstra, P. S.; Frejafon, E.; Hubert, P.; Gribaldo, L.; Kearns, P.; Aublant, J.-M.; Diabaté, S.; Weiss, C.; de Groot, A.; Kooter, I., Air-Liquid Interface In Vitro Models for Respiratory Toxicology Research: Consensus Workshop and Recommendations. *Applied In Vitro Toxicology* **2018**, 4 (2), 91-106.
5. Cruz, A. A., *Global surveillance, prevention and control of chronic respiratory diseases: a comprehensive approach*. World Health Organization: 2007.
6. National Institutes of, H., Estimates of funding for various research, condition, and disease categories (RCDC). See <http://report.nih.gov/rcdc/categories/> (last accessed 17 February 2012) **2017**.
7. Andersen, K. G.; Rambaut, A.; Lipkin, W. I.; Holmes, E. C.; Garry, R. F., The proximal origin of SARS-CoV-2. *Nature medicine* **2020**, 26 (4), 450-452.
8. Zhou, P.; Yang, X.-L.; Wang, X.-G.; Hu, B.; Zhang, L.; Zhang, W.; Si, H.-R.; Zhu, Y.; Li, B.; Huang, C.-L., A pneumonia outbreak associated with a new coronavirus of probable bat origin. *nature* **2020**, 579 (7798), 270-273.
9. Marois, G.; Muttarak, R.; Scherbov, S., Assessing the potential impact of COVID-19 on life expectancy. *PloS one* **2020**, 15 (9), e0238678.
10. Sarkodie, S. A.; Owusu, P. A., Global assessment of environment, health and economic impact of the novel coronavirus (COVID-19). *Environment, Development and Sustainability* **2021**, 23, 5005-5015.
11. Pak, A.; Adegboye, O. A.; Adekunle, A. I.; Rahman, K. M.; McBryde, E. S.; Eisen, D. P., Economic consequences of the COVID-19 outbreak: the need for epidemic preparedness. *Frontiers in public health* **2020**, 8, 241.
12. Yamey, G.; Schäferhoff, M.; Pate, M.; Chawla, M.; Ranson, K.; Hatchett, R.; Wilder, R., Funding the development and manufacturing of COVID-19 vaccines. *The Center for Policy Impact in Global Health., Duke Global Working Paper Series* **2020**, (20).
13. Movia, D.; Prina-Mello, A., Preclinical Development of Orally Inhaled Drugs (OIDs)—Are Animal Models Predictive or Shall We Move Towards In Vitro Non-Animal Models? *Animals* **2020**, 10 (8), 1259.
14. Brigden, G.; du Cros, P.; Wong, S., Barriers to new drug development in respiratory disease. *European Respiratory Journal* **2016**, 47 (1), 356-357.
15. Barnes, P. J.; Bonini, S.; Seeger, W.; Belvisi, M. G.; Ward, B.; Holmes, A., Barriers to new drug development in respiratory disease. *European Respiratory Journal* **2015**, 45 (5), 1197.
16. O'Leary, C.; Gilbert, J. L.; O'Dea, S.; O'Brien, F. J.; Cryan, S. A., Respiratory Tissue Engineering: Current Status and Opportunities for the Future. *Tissue engineering. Part B, Reviews* **2015**, 21 (4), 323-44.
17. Edwards, D. A.; Dunbar, C., Bioengineering of therapeutic aerosols. *Annual review of biomedical engineering* **2002**, 4 (1), 93-107.
18. Jensen, G.; Morrill, C.; Huang, Y., 3D tissue engineering, an emerging technique for pharmaceutical research. *Acta Pharmaceutica Sinica B* **2018**, 8 (5), 756-766.
19. Nossa, R.; Costa, J.; Cacopardo, L.; Ahluwalia, A., Breathing in vitro: Designs and applications of engineered lung models. *Journal of Tissue Engineering* **2021**, 12, 20417314211008696.
20. Faber, S. C.; McCullough, S. D., Through the looking glass: in vitro models for inhalation toxicology and interindividual variability in the airway. *Applied in vitro toxicology* **2018**, 4 (2), 115-128.
21. Prakash, Y. S.; Tschumperlin, D. J.; Stenmark, K. R., Coming to terms with tissue engineering and regenerative medicine in the lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **2015**, 309 (7), L625-L638.
22. Acocella, F.; Brizzola, S., 12 - Tracheal tissue regeneration. In *Electrospinning for Tissue Regeneration*, Bosworth, L. A.; Downes, S., Eds. Woodhead Publishing: 2011; pp 242-279.
23. Placone, J. K.; Mahadik, B.; Fisher, J. P., Addressing present pitfalls in 3D printing for tissue engineering to enhance future potential. *APL bioengineering* **2020**, 4 (1), 010901.
24. Williams, D., Benefit and risk in tissue engineering. *Materials Today* **2004**, 7 (5), 24-29.

25. O'Leary, C.; Soriano, L.; Fagan-Murphy, A.; Ivankovic, I.; Cavanagh, B.; O'Brien, F. J.; Cryan, S.-A., The Fabrication and in vitro Evaluation of Retinoic Acid-Loaded Electrospun Composite Biomaterials for Tracheal Tissue Regeneration. *Frontiers in bioengineering and biotechnology* **2020**, *8*, 190.
26. Khan, Y. S.; Lynch, D. T., Histology, Lung. In *StatPearls*, StatPearls Publishing

Copyright © 2021, StatPearls Publishing LLC.: Treasure Island (FL), 2021.

27. Mescher, A. L., *Junqueira's basic histology: text and atlas*. McGraw-Hill Medical 13th ed. New York: 2013; Vol. 12.
28. Patwa, A.; Shah, A., Anatomy and physiology of respiratory system relevant to anaesthesia. *Indian journal of anaesthesia* **2015**, *59* (9), 533.
29. Cardoso, W. V.; Kotton, D. N., Specification and patterning of the respiratory system. In *StemBook*, Harvard Stem Cell Institute

Copyright: © 2008 Wellington V. Cardoso and Darrell N. Kotton.: Cambridge (MA), 2008.

30. Weupe, M.; Peabody Lever, J. E.; Kennemur, J. P.; Bono, T. R.; Phillips, S. E.; Shei, R.-J.; Rowe, S. M., Moving Mucus Matters for Lung Health. *Frontiers for Young Minds* **2019**, *7*.
31. Fröhlich, E., Comparison of conventional and advanced in vitro models in the toxicity testing of nanoparticles. *Artificial Cells, Nanomedicine, and Biotechnology* **2018**, *46* (sup2), 1091-1107.
32. Nettekheim, P.; Koo, J. S.; Gray, T., Regulation of differentiation of the tracheobronchial epithelium. *Journal of aerosol medicine* **2000**, *13* (3), 207-218.
33. Delaere, P., Tracheal replacement: state of the art and novel perspectives. *J Vis Surg* **2018**, *4*, 168.
34. Iwasaki, A.; Foxman, E. F.; Molony, R. D., Early local immune defences in the respiratory tract. *Nature Reviews Immunology* **2017**, *17* (1), 7-20.
35. Boazak, E. M.; Auguste, D. T., Trachea Mechanics for Tissue Engineering Design. *ACS Biomaterials Science & Engineering* **2018**, *4* (4), 1272-1284.
36. Furlow, P. W.; Mathisen, D. J., Surgical anatomy of the trachea. *Annals of cardiothoracic surgery* **2018**, *7* (2), 255.
37. Brand-Saberi, B. E.; Schäfer, T., Trachea: anatomy and physiology. *Thorac Surg Clin* **2014**, *24* (1), 1-5.
38. Teng, Z.; Trabelsi, O.; Ochoa, I.; He, J.; Gillard, J. H.; Doblare, M. J. J. o. b., Anisotropic material behaviours of soft tissues in human trachea: an experimental study. **2012**, *45* (9), 1717-1723.
39. Kaye, R.; Green, G. E.; Smith, L. P., Tracheal Replacement. In *Encyclopedia of Tissue Engineering and Regenerative Medicine*, Reis, R. L., Ed. Academic Press: Oxford, 2019; pp 281-284.
40. Plumlee, K. H., Chapter 15 - Respiratory System. In *Clinical Veterinary Toxicology*, Plumlee, K. H., Ed. Mosby: Saint Louis, 2004; pp 80-88.
41. Prange, T., Chapter 48 - Trachea. In *Equine Surgery (Fifth Edition)*, Auer, J. A.; Stick, J. A.; Kümmeler, J. M.; Prange, T., Eds. W.B. Saunders: 2019; pp 797-804.
42. Böck, P.; Stockinger, L., Light and electron microscopic identification of elastic, elastin and oxytalan fibers in human tracheal and bronchial mucosa. *Anatomy and embryology* **1984**, *170* (2), 145-153.
43. Hardie, E., Chapter 46 - Trachea and bronchus. In *Feline Soft Tissue and General Surgery*, Langley-Hobbs, S. J.; Demetriou, J. L.; Ladlow, J. F., Eds. W.B. Saunders: 2014; pp 531-540.
44. Fernandez, L. G.; Norwood, S. H.; Berne, J. D., CHAPTER 32 - TRACHEAL, LARYNGEAL, AND OROPHARYNGEAL INJURIES. In *Current Therapy of Trauma and Surgical Critical Care*, Asensio, J. A.; Trunkey, D. D., Eds. Mosby: Philadelphia, 2008; pp 215-226.
45. Knight, D. A.; Holgate, S. T., The airway epithelium: structural and functional properties in health and disease. *Respirology (Carlton, Vic.)* **2003**, *8* (4), 432-46.
46. Wilmott, R. W.; Bush, A.; Deterding, R. R.; Ratjen, F.; Sly, P.; Zar, H.; Li, A., *Kendig's disorders of the respiratory tract in children e-book*. Elsevier Health Sciences: 2018.
47. Corfield, A. P., Mucins: A biologically relevant glycan barrier in mucosal protection. *Biochimica et Biophysica Acta (BBA) - General Subjects* **2015**, *1850* (1), 236-252.
48. Rogers, D. F., Airway goblet cells: responsive and adaptable front-line defenders. *The European respiratory journal* **1994**, *7* (9), 1690-706.
49. Gilpin, S. E.; Charest, J. M.; Ren, X.; Tapias, L. F.; Wu, T.; Evangelista-Leite, D.; Mathisen, D. J.; Ott, H. C., Regenerative potential of human airway stem cells in lung epithelial engineering. *Biomaterials* **2016**, *108*, 111-9.
50. Xing, Y.; Li, C.; Li, A.; Sridurongrit, S.; Tiozzo, C.; Bellusci, S.; Borok, Z.; Kaartinen, V.; Minoo, P., Signaling via Alk5 controls the ontogeny of lung Clara cells. *Development* **2010**, *137* (5), 825-833.

51. Hewitt, R. J.; Lloyd, C. M., Regulation of immune responses by the airway epithelial cell landscape. *Nature Reviews Immunology* **2021**, *21* (6), 347-362.
52. Davis, J. D.; Wypych, T. P., Cellular and functional heterogeneity of the airway epithelium. *Mucosal Immunology* **2021**, 1-13.
53. Montoro, D. T.; Haber, A. L.; Biton, M.; Vinarsky, V.; Lin, B.; Birket, S. E.; Yuan, F.; Chen, S.; Leung, H. M.; Villoria, J., A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature* **2018**, *560* (7718), 319-324.
54. Wachsmuth, L.; Söder, S.; Fan, Z.; Finger, F.; Aigner, T. J. H.; histopathology, Immunolocalization of matrix proteins in different human cartilage subtypes. **2006**.
55. Kelly, D. J.; Crawford, A.; Dickinson, S. C.; Sims, T. J.; Mundy, J.; Hollander, A. P.; Prendergast, P. J.; Hatton, P. V., Biochemical markers of the mechanical quality of engineered hyaline cartilage. *Journal of Materials Science: Materials in Medicine* **2007**, *18* (2), 273-281.
56. Şenol, M. S.; Özer, H., Architecture of cartilage tissue and its adaptation to pathological conditions. In *Comparative Kinesiology of the Human Body*, Elsevier: 2020; pp 91-100.
57. Fuss, M.; Ehlers, E. M.; Russlies, M.; Rohwedel, J.; Behrens, P., Characteristics of human chondrocytes, osteoblasts and fibroblasts seeded onto a type I/III collagen sponge under different culture conditions: a light, scanning and transmission electron microscopy study. *Annals of Anatomy-Anatomischer Anzeiger* **2000**, *182* (4), 303-310.
58. Chang, L.-R.; Marston, G.; Martin, A., Anatomy, cartilage. *StatPearls [Internet]* **2020**.
59. Hall, J. E.; Hall, M. E., *Guyton and Hall textbook of medical physiology e-Book*. Elsevier Health Sciences: 2020.
60. Reynolds, S. D.; Pinkerton, K. E.; Mariassy, A. T., Epithelial cells of trachea and bronchi. In *Comparative biology of the Normal lung*, Elsevier: 2015; pp 61-81.
61. Macklem, P. T.; Mead, J., Factors determining maximum expiratory flow in dogs. *Journal of applied physiology* **1968**, *25* (2), 159-169.
62. Mow, V. C.; Kuei, S.; Lai, W. M.; Armstrong, C. G. J. J. o. b. e., Biphasic creep and stress relaxation of articular cartilage in compression: theory and experiments. **1980**, *102* (1), 73-84.
63. Safshekan, F.; Tafazzoli-Shadpour, M.; Abdouss, M.; Shadmehr, M. B. J. M., Mechanical characterization and constitutive modeling of human trachea: age and gender dependency. **2016**, *9* (6), 456.
64. Sarma, P.; Pidaparti, R.; Meiss, R. J. J. o. B. M. R. P. A. A. O. J. o. T. S. f. B., The Japanese Society for Biomaterials.; Biomaterials, T. A. S. f.; Biomaterials, t. K. S. f., Anisotropic properties of tracheal smooth muscle tissue. **2003**, *65* (1), 1-8.
65. Wang, J.-Y.; Mesquida, P.; Pallai, P.; Corrigan, C. J.; Lee, T. H. J. a. p. a., Dynamic properties of human bronchial airway tissues. **2011**.
66. Hoffman, B.; Martin, M.; Brown, B. N.; Bonassar, L. J.; Cheetham, J. J. T. L., Biomechanical and biochemical characterization of porcine tracheal cartilage. **2016**, *126* (10), E325-E331.
67. Karkhanis, T.; Rao, M.; Zafar, F.; Simon Morales, D. L.; Haridas, B., Tracheal Cartilage Ring Biomechanical Properties for Pediatric Exostent Design. *Journal of Medical Devices* **2016**, *10* (2).
68. Rains, J.; Bert, J.; Roberts, C.; Pare, P. J. J. o. A. P., Mechanical properties of human tracheal cartilage. **1992**, *72* (1), 219-225.
69. Roberts, C. R.; Rains, J. K.; Paré, P. D.; Walker, D. C.; Wiggs, B.; Bert, J. L. J. J. o. b., Ultrastructure and tensile properties of human tracheal cartilage. **1997**, *31* (1), 81-86.
70. Trabelsi, O.; Del Palomar, A. P.; López-Villalobos, J.; Ginel, A.; Doblaré, M. J. M. e.; physics, Experimental characterization and constitutive modeling of the mechanical behavior of the human trachea. **2010**, *32* (1), 76-82.
71. Lambert, R.; Baile, E.; Moreno, R.; Bert, J.; Paré, P. J. J. o. A. P., A method for estimating the Young's modulus of complete tracheal cartilage rings. **1991**, *70* (3), 1152-1159.
72. Etienne, H.; Fabre, D.; Gomez Caro, A.; Kolb, F.; Mussot, S.; Mercier, O.; Mitilian, D.; Stephan, F.; Fadel, E.; Darteville, P., Tracheal replacement. *The European respiratory journal* **2018**, *51* (2).
73. Kojima, K.; Vacanti, C. A. J. T. A. R., Tissue engineering in the trachea. **2014**, *297* (1), 44-50.
74. Mansfield, E. G.; Greene, V. K., Jr.; Auguste, D. T., Patterned, tubular scaffolds mimic longitudinal and radial mechanics of the neonatal trachea. *Acta biomaterialia* **2016**, *33*, 176-82.
75. Sun, F.; Jiang, Y.; Xu, Y.; Shi, H.; Zhang, S.; Liu, X.; Pan, S.; Ye, G.; Zhang, W.; Zhang, F. J. S. r., Genipin cross-linked decellularized tracheal tubular matrix for tracheal tissue engineering applications. **2016**, *6* (1), 1-12.
76. Park, H. S.; Lee, J. S.; Jung, H.; Kim, D. Y.; Kim, S. W.; Sultan, M. T.; Park, C. H. J. A. c., nanomedicine.; biotechnology, An omentum-cultured 3D-printed artificial trachea: in vivo bioreactor. **2018**, *46* (sup3), S1131-S1140.

77. Ramos, T.; Moroni, L., Tissue Engineering and Regenerative Medicine 2019: The Role of Biofabrication—A Year in Review. *Tissue Engineering Part C: Methods* **2019**, 26 (2), 91-106.
78. Almouemen, N.; Kelly, H. M.; O'Leary, C., Tissue engineering: understanding the role of biomaterials and biophysical forces on cell functionality through computational and structural biotechnology analytical methods. *Computational and structural biotechnology journal* **2019**, 17, 591-598.
79. Mhanna, R.; Hasan, A., Introduction to tissue engineering. *Tissue Engineering for Artificial Organs* **2017**, 1-34.
80. De Isla, N.; Huseltein, C.; Jessel, N.; Pinzano, A.; Decot, V.; Magdalou, J.; Bensoussan, D.; Stoltz, J. F., Introduction to tissue engineering and application for cartilage engineering. *Bio-medical materials and engineering* **2010**, 20 (3-4), 127-133.
81. Sundarakrishnan, A.; Chen, Y.; Black, L. D.; Aldridge, B. B.; Kaplan, D. L., Engineered cell and tissue models of pulmonary fibrosis. *Advanced drug delivery reviews* **2018**, 129, 78-94.
82. Tebyanian, H.; Karami, A.; Nourani, M. R.; Motavallian, E.; Barkhordari, A.; Yazdanian, M.; Seifalian, A., Lung tissue engineering: An update. *Journal of cellular physiology* **2019**, 234 (11), 19256-19270.
83. Forbes, B., Human airway epithelial cell lines for in vitro drug transport and metabolism studies. *Pharmaceutical science & technology today* **2000**, 3 (1), 18-27.
84. Hamilton, N. J. I.; Lee, D. D. H.; Gowers, K. H. C.; Butler, C. R.; Maughan, E. F.; Jevans, B.; Orr, J. C.; McCann, C. J.; Burns, A. J.; MacNeil, S., Bioengineered airway epithelial grafts with mucociliary function based on collagen IV-and laminin-containing extracellular matrix scaffolds. *European Respiratory Journal* **2020**, 55 (6).
85. Boys, A. J.; Barron, S. L.; Tilev, D.; Owens, R. M., Building Scaffolds for Tubular Tissue Engineering. *Frontiers in Bioengineering and Biotechnology* **2020**, 8, 1357.
86. Soriano, L.; Khalid, T.; O'Brien, F. J.; O'Leary, C.; Cryan, S.-A., A Tissue-Engineered Tracheobronchial In Vitro Co-Culture Model for Determining Epithelial Toxicological and Inflammatory Responses. *Biomedicines* **2021**, 9 (6), 631.
87. Sousa, F.; Castro, P., Cell-based in vitro models for nasal permeability studies. In *Concepts and Models for Drug Permeability Studies*, Elsevier: 2016; pp 83-100.
88. Ong, H. X.; Traini, D.; Young, P. M., Pharmaceutical applications of the Calu-3 lung epithelia cell line. *Expert opinion on drug delivery* **2013**, 10 (9), 1287-1302.
89. Forbes, B.; Shah, A.; Martin, G. P.; Lansley, A. B., The human bronchial epithelial cell line 16HBE14o- as a model system of the airways for studying drug transport. *International journal of pharmaceutics* **2003**, 257 (1-2), 161-167.
90. Callaghan, P.; Ferrick, B.; Rybakovsky, E.; Thomas, S.; Mullin, J., Epithelial Barrier Properties of the 16HBE14o-Human Bronchial Epithelial Cell Culture Model. *Bioscience Reports* **2020**.
91. Hermanns, M. I.; Freese, C.; Anspach, L.; Grützner, V.; Pohl, C.; Unger, R. E.; Kirkpatrick, C. J., 3.15 Cell Culture Systems for Studying Biomaterial Interactions With Biological Barriers. **2017**.
92. Katsumiti, A.; Ruenaroengsak, P.; Cajaville, M. P.; Thorley, A. J.; Tetley, T. D., Immortalisation of primary human alveolar epithelial lung cells using a non-viral vector to study respiratory bioreactivity in vitro. *Scientific reports* **2020**, 10 (1), 1-13.
93. Hiemstra, P. S.; Tetley, T. D.; Janes, S. M., Airway and alveolar epithelial cells in culture. *European Respiratory Journal* **2019**, 54 (5).
94. Rayner, R. E.; Makena, P.; Prasad, G. L.; Cormet-Boyaka, E., Optimization of normal human bronchial epithelial (NHBE) cell 3D cultures for in vitro lung model studies. *Scientific reports* **2019**, 9 (1), 1-10.
95. Kim, I. G.; Park, S. A.; Lee, S.-H.; Choi, J. S.; Cho, H.; Lee, S. J.; Kwon, Y.-W.; Kwon, S. K., Transplantation of a 3D-printed tracheal graft combined with iPS cell-derived MSCs and chondrocytes. *Scientific reports* **2020**, 10 (1), 1-14.
96. Butler, C. R.; Hynds, R. E.; Crowley, C.; Gowers, K. H. C.; Partington, L.; Hamilton, N. J.; Carvalho, C.; Platé, M.; Samuel, E. R.; Burns, A. J., Vacuum-assisted decellularization: an accelerated protocol to generate tissue-engineered human tracheal scaffolds. *Biomaterials* **2017**, 124, 95-105.
97. Park, J.-H.; Yoon, J.-K.; Lee, J. B.; Shin, Y. M.; Lee, K.-W.; Bae, S.-W.; Lee, J.; Yu, J.; Jung, C.-R.; Youn, Y.-N., Experimental tracheal replacement using 3-dimensional bioprinted artificial trachea with autologous epithelial cells and chondrocytes. *Scientific reports* **2019**, 9 (1), 1-11.
98. Wark, P. A. B.; Johnston, S. L.; Bucchieri, F.; Powell, R.; Puddicombe, S.; Laza-Stanca, V.; Holgate, S. T.; Davies, D. E., Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *The Journal of experimental medicine* **2005**, 201 (6), 937-947.

99. Rayner, R. E.; Wellmerling, J.; Osman, W.; Honesty, S.; Alfaro, M.; Peeples, M. E.; Cormet-Boyaka, E., In vitro 3D culture lung model from expanded primary cystic fibrosis human airway cells. *Journal of Cystic Fibrosis* **2020**, *19* (5), 752-761.
100. Roomans, G. M. J. E. C. M., Tissue engineering and the use of stem/progenitor cells for airway epithelium repair. **2010**, *19* (284), 99.
101. He, X.; Fu, W.; Zheng, J., Cell sources for trachea tissue engineering: past, present and future. *Regenerative medicine* **2012**, *7* (6), 851-863.
102. Komura, M.; Komura, H.; Tanaka, Y.; Kanamori, Y.; Sugiyama, M.; Nakahara, S.; Kawashima, H.; Suzuki, K.; Hoshi, K.; Iwanaka, T., Human tracheal chondrocytes as a cell source for augmenting stenotic tracheal segments: the first feasibility study in an in vivo culture system. *Pediatric surgery international* **2008**, *24* (10), 1117-1121.
103. Schulze-Tanzil, G., Activation and dedifferentiation of chondrocytes: implications in cartilage injury and repair. *Annals of Anatomy-Anatomischer Anzeiger* **2009**, *191* (4), 325-338.
104. Imaizumi, M.; Nomoto, Y.; Sato, Y.; Sugino, T.; Miyake, M.; Wada, I.; Nakamura, T.; Omori, K., Evaluation of the use of induced pluripotent stem cells (iPSCs) for the regeneration of tracheal cartilage. *Cell transplantation* **2013**, *22* (2), 341-353.
105. Taniguchi, D.; Matsumoto, K.; Machino, R.; Takeoka, Y.; Elgalad, A.; Taura, Y.; Oyama, S.; Tetsuo, T.; Moriyama, M.; Takagi, K., Human lung microvascular endothelial cells as potential alternatives to human umbilical vein endothelial cells in bio-3D-printed trachea-like structures. *Tissue and Cell* **2020**, *63*, 101321.
106. Harrington, H.; Cato, P.; Salazar, F.; Wilkinson, M.; Knox, A.; Haycock, J. W.; Rose, F.; Aylott, J. W.; Ghaemmaghami, A. M., Immunocompetent 3D Model of Human Upper Airway for Disease Modeling and In Vitro Drug Evaluation. *Molecular Pharmaceutics* **2014**, *11* (7), 2082-2091.
107. Zhu, Y.; Chidekel, A.; Shaffer, T. H., Cultured human airway epithelial cells (calu-3): a model of human respiratory function, structure, and inflammatory responses. *Critical care research and practice* **2010**, *2010*.
108. Jesus, S.; Schmutz, M.; Som, C.; Borchard, G.; Wick, P.; Borges, O., Hazard assessment of polymeric nanobiomaterials for drug delivery: what can we learn from literature so far. *Frontiers in bioengineering and biotechnology* **2019**, *7*, 261.
109. Garcia-Canton, C.; Minet, E.; Anadon, A.; Meredith, C., Metabolic characterization of cell systems used in in vitro toxicology testing: lung cell system BEAS-2B as a working example. *Toxicology in Vitro* **2013**, *27* (6), 1719-1727.
110. Han, X.; Na, T.; Wu, T.; Yuan, B.-Z., Human lung epithelial BEAS-2B cells exhibit characteristics of mesenchymal stem cells. *PloS one* **2020**, *15* (1), e0227174.
111. Davis, A. S.; Chertow, D. S.; Moyer, J. E.; Suzich, J.; Sandouk, A.; Dorward, D. W.; Logun, C.; Shelhamer, J. H.; Taubenberger, J. K., Validation of normal human bronchial epithelial cells as a model for influenza A infections in human distal trachea. *Journal of Histochemistry & Cytochemistry* **2015**, *63* (5), 312-328.
112. Kesimer, M.; Kirkham, S.; Pickles, R. J.; Henderson, A. G.; Alexis, N. E.; DeMaria, G.; Knight, D.; Thornton, D. J.; Sheehan, J. K., Tracheobronchial air-liquid interface cell culture: a model for innate mucosal defense of the upper airways? *American Journal of Physiology-Lung Cellular and Molecular Physiology* **2009**, *296* (1), L92-L100.
113. Eckerle, I.; Müller, M. A.; Kallies, S.; Gotthardt, D. N.; Drosten, C., In-vitro renal epithelial cell infection reveals a viral kidney tropism as a potential mechanism for acute renal failure during Middle East Respiratory Syndrome (MERS) Coronavirus infection. *Virology journal* **2013**, *10* (1), 1-5.
114. Leung, C.; Wadsworth, S. J.; Yang, S. J.; Dorscheid, D. R., Structural and functional variations in human bronchial epithelial cells cultured in air-liquid interface using different growth media. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **2020**, *318* (5), L1063-L1073.
115. Sungnak, W.; Huang, N.; Bécavin, C.; Berg, M.; Queen, R.; Litvinukova, M.; Talavera-López, C.; Maatz, H.; Reichart, D.; Sampaziotis, F., SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. *Nature medicine* **2020**, *26* (5), 681-687.
116. Ulm, A.; Mayhew, C. N.; Debley, J.; Hershey, G. K. K.; Ji, H., Cultivate primary nasal epithelial cells from children and reprogram into induced pluripotent stem cells. *Journal of visualized experiments: JoVE* **2016**, (109).
117. McDougall, C. M.; Blaylock, M. G.; Douglas, J. G.; Brooker, R. J.; Helms, P. J.; Walsh, G. M., Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. *American journal of respiratory cell and molecular biology* **2008**, *39* (5), 560-568.
118. Ziegelaar, B. W.; Aigner, J.; Staudenmaier, R.; Lempart, K.; Mack, B.; Happ, T.; Sittlinger, M.; Endres, M.; Naumann, A.; Kastenbauer, E., The characterisation of human respiratory epithelial

- cells cultured on resorbable scaffolds: first steps towards a tissue engineered tracheal replacement. *Biomaterials* **2002**, 23 (6), 1425-1438.
119. Agu, R. U.; Jorissen, M.; Willems, T.; de Witte, P.; Kinget, R.; Verbeke, N., Derivatized and fibrillar collagen films as scaffold for nasal epithelial cells to study nasal drug absorption in vitro. *STP pharma sciences* **2002**, 12 (1), 81-87.
 120. Coraux, C.; Nawrocki-Raby, B.; Hinnrasky, J.; Kileztky, C.; Gaillard, D.; Dani, C.; Puchelle, E., Embryonic stem cells generate airway epithelial tissue. *American journal of respiratory cell and molecular biology* **2005**, 32 (2), 87-92.
 121. Le Suer, J.; Sease, R.; Hawkins, F.; Ryan, A. L., Induced pluripotent stem cells for generating lung airway stem cells and modelling respiratory disease. *Lung Stem Cells in Development, Health and Disease (ERS Monograph)*. Sheffield, European Respiratory Society **2021**, 190-204.
 122. Kawakita, N.; Toba, H.; Miyoshi, K.; Sakamoto, S.; Matsumoto, D.; Takashima, M.; Aoyama, M.; Inoue, S.; Morimoto, M.; Nishino, T., Bronchioalveolar stem cells derived from mouse-induced pluripotent stem cells promote airway epithelium regeneration. *Stem Cell Research & Therapy* **2020**, 11 (1), 1-14.
 123. Wang, R.; McCauley, K. B.; Kotton, D. N.; Hawkins, F., Differentiation of human airway-organoids from induced pluripotent stem cells (iPSCs). *Methods in Cell Biology* **2020**, 159, 95-114.
 124. Azargoon, A.; Negahdari, B., Lung regeneration using amniotic fluid mesenchymal stem cells. *Artificial cells, nanomedicine, and biotechnology* **2018**, 46 (3), 447-451.
 125. Sarkar, K.; Xue, Y.; Sant, S., Host response to synthetic versus natural biomaterials. In *The Immune Response to Implanted Materials and Devices*, Springer: 2017; pp 81-105.
 126. Giannitelli, S. M.; Accoto, D.; Trombetta, M.; Rainer, A., Current trends in the design of scaffolds for computer-aided tissue engineering. *Acta biomaterialia* **2014**, 10 (2), 580-594.
 127. Lim, Y.-S.; Ok, Y.-J.; Hwang, S.-Y.; Kwak, J.-Y.; Yoon, S., Marine collagen as a promising biomaterial for biomedical applications. *Marine drugs* **2019**, 17 (8), 467.
 128. Reddy, M.; Ponnamm, D.; Choudhary, R.; Sadasivuni, K. K., A comparative review of natural and synthetic biopolymer composite scaffolds. *Polymers* **2021**, 13 (7), 1105.
 129. Laurencin, C.; Deng, M., *Natural and synthetic biomedical polymers*. Newnes: 2014.
 130. Prasadh, S.; Wong, R. C. W., Unraveling the mechanical strength of biomaterials used as a bone scaffold in oral and maxillofacial defects. *Oral Science International* **2018**, 15 (2), 48-55.
 131. Hamilton, N. J. I.; Hynds, R. E.; Gowers, K. H. C.; Tait, A.; Butler, C. R.; Hopper, C.; Burns, A. J.; Birchall, M. A.; Lowdell, M.; Janes, S. M., Using a three-dimensional collagen matrix to deliver respiratory progenitor cells to decellularized trachea in vivo. *Tissue Engineering Part C: Methods* **2019**, 25 (2), 93-102.
 132. O'Leary, C.; Cavanagh, B.; Unger, R. E.; Kirkpatrick, C. J.; O'Dea, S.; O'Brien, F. J.; Cryan, S.-A., The development of a tissue-engineered tracheobronchial epithelial model using a bilayered collagen-hyaluronate scaffold. *Biomaterials* **2016**, 85, 111-127.
 133. Wang, L.; Zhao, Y.; Yang, F.; Feng, M.; Zhao, Y.; Chen, X.; Mi, J.; Yao, Y.; Guan, D.; Xiao, Z., Biomimetic collagen biomaterial induces in situ lung regeneration by forming functional alveolar. *Biomaterials* **2020**, 236, 119825.
 134. Chen, P.; Marsilio, E.; Goldstein, R. H.; Yannas, I. V.; Spector, M., Formation of lung alveolar-like structures in collagen-glycosaminoglycan scaffolds in vitro. *Tissue engineering* **2005**, 11 (9-10), 1436-1448.
 135. Wang, L.; Guo, X.; Chen, J.; Zhen, Z.; Cao, B.; Wan, W.; Dou, Y.; Pan, H.; Xu, F.; Zhang, Z., Key considerations on the development of biodegradable biomaterials for clinical translation of medical devices: With cartilage repair products as an example. *Bioactive Materials* **2021**.
 136. Kobayashi, K.; Suzuki, T.; Nomoto, Y.; Tada, Y.; Miyake, M.; Hazama, A.; Wada, I.; Nakamura, T.; Omori, K., A tissue-engineered trachea derived from a framed collagen scaffold, gingival fibroblasts and adipose-derived stem cells. *Biomaterials* **2010**, 31 (18), 4855-4863.
 137. Nakaegawa, Y.; Nakamura, R.; Tada, Y.; Nomoto, Y.; Imaizumi, M.; Suzuki, R.; Nakamura, T.; Omori, K., Effect of structural differences in collagen sponge scaffolds on tracheal epithelium regeneration. *Annals of Otolaryngology, Rhinology & Laryngology* **2016**, 125 (2), 115-122.
 138. Nakamura, R.; Tani, A.; Yoshie, S.; Ikeda, M.; Wada, I.; Hazama, A.; Nomoto, Y.; Tada, Y.; Nakamura, T.; Omori, K., Heparin cross-linked collagen sponge scaffolds improve functional regeneration of rat tracheal epithelium. *Journal of tissue engineering and regenerative medicine* **2017**, 11 (11), 3027-3037.
 139. Choi, J. S.; Lee, M. S.; Kim, J.; Eom, M. R.; Jeong, E. J.; Lee, M.; Park, S. A.; Jeong, J. H.; Kwon, S. K., Hyaluronic Acid Coating on Hydrophobic Tracheal Scaffold Enhances Mesenchymal Stem Cell Adhesion and Tracheal Regeneration. *Tissue Engineering and Regenerative Medicine* **2021**, 18 (2), 225-233.

140. Drago, L.; Cappelletti, L.; De Vecchi, E.; Pignataro, L.; Torretta, S.; Mattina, R., Antiadhesive and antibiofilm activity of hyaluronic acid against bacteria responsible for respiratory tract infections. *Apms* **2014**, *122* (10), 1013-1019.
141. Bridge, J. C.; Aylott, J. W.; Brightling, C. E.; Ghaemmaghami, A. M.; Knox, A. J.; Lewis, M. P.; Rose, F. R. A. J.; Morris, G. E., Adapting the electrospinning process to provide three unique environments for a tri-layered in vitro model of the airway wall. *Journal of visualized experiments: JoVE* **2015**, (101).
142. Teijeiro-Osorio, D.; Remuñán-López, C.; Alonso, M. J., Chitosan/cyclodextrin nanoparticles can efficiently transfect the airway epithelium in vitro. *European Journal of Pharmaceutics and Biopharmaceutics* **2009**, *71* (2), 257-263.
143. Romanova, O. A.; Tenchurin, T. H.; Demina, T. S.; Sytina, E. V.; Shepelev, A. D.; Rudyak, S. G.; Klein, O. I.; Krashennnikov, S. V.; Safronova, E. I.; Kamyshinsky, R. A., Non-woven bilayered biodegradable chitosan-gelatin-poly lactide scaffold for bioengineering of tracheal epithelium. *Cell proliferation* **2019**, *52* (3), e12598.
144. Tatekawa, Y.; Kawazoe, N.; Chen, G.; Shirasaki, Y.; Komuro, H.; Kaneko, M., Tracheal defect repair using a PLGA-collagen hybrid scaffold reinforced by a copolymer stent with bFGF-impregnated gelatin hydrogel. *Pediatric surgery international* **2010**, *26* (6), 575-580.
145. Thompson, H. G.; Truong, D. T.; Griffith, C. K.; George, S. C., A three-dimensional in vitro model of angiogenesis in the airway mucosa. *Pulmonary pharmacology & therapeutics* **2007**, *20* (2), 141-148.
146. Kumar, P.; Ciftci, S.; Barthes, J.; Knopf-Marques, H.; Muller, C. B.; Debry, C.; Vrana, N. E.; Ghaemmaghami, A. M., A composite Gelatin/hyaluronic acid hydrogel as an ECM mimic for developing mesenchymal stem cell-derived epithelial tissue patches. *Journal of tissue engineering and regenerative medicine* **2020**, *14* (1), 45-57.
147. Cornelissen, C. G.; Dietrich, M.; Krüger, S.; Spillner, J.; Schmitz-Rode, T.; Jockenhoevel, S., Fibrin gel as alternative scaffold for respiratory tissue engineering. *Annals of biomedical engineering* **2012**, *40* (3), 679-687.
148. Chen, Z.; Zhong, N.; Wen, J.; Jia, M.; Guo, Y.; Shao, Z.; Zhao, X., Porous three-dimensional silk fibroin scaffolds for tracheal epithelial regeneration in vitro and in vivo. *ACS Biomaterials Science & Engineering* **2018**, *4* (8), 2977-2985.
149. Zhu, J., Bioactive modification of poly (ethylene glycol) hydrogels for tissue engineering. *Biomaterials* **2010**, *31* (17), 4639-4656.
150. Burg, K., Poly (α -ester) s. In *Natural and Synthetic Biomedical Polymers*, Elsevier: 2014; pp 115-121.
151. Guo, B.; Ma, P. X., Synthetic biodegradable functional polymers for tissue engineering: a brief review. *Science China Chemistry* **2014**, *57* (4), 490-500.
152. Cheow, W. S.; Ng, M. L. L.; Kho, K.; Hadinoto, K., Spray-freeze-drying production of thermally sensitive polymeric nanoparticle aggregates for inhaled drug delivery: effect of freeze-drying adjuvants. *International journal of pharmaceutics* **2011**, *404* (1-2), 289-300.
153. Meenach, S. A.; Vogt, F. G.; Anderson, K. W.; Hilt, J. Z.; McGarry, R. C.; Mansour, H. M., Design, physicochemical characterization, and optimization of organic solution advanced spray-dried inhalable dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine poly (ethylene glycol)(DPPE-PEG) microparticles and nanoparticles for targeted respiratory nanomedicine delivery as dry powder inhalation aerosols. *International journal of nanomedicine* **2013**, *8*, 275.
154. Vij, N., Synthesis and evaluation of airway targeted PLGA nanoparticles for drug delivery in obstructive lung diseases. In *Nanoparticles in Biology and Medicine*, Springer: 2012; pp 303-310.
155. Bogan, S. L.; Teoh, G. Z.; Birchall, M. A., Tissue engineered airways: a prospects article. *Journal of cellular biochemistry* **2016**, *117* (7), 1497-1505.
156. Ghorbani, F.; Moradi, L.; Shadmehr, M. B.; Bonakdar, S.; Droodinia, A.; Safshekan, F., In-vivo characterization of a 3D hybrid scaffold based on PCL/decellularized aorta for tracheal tissue engineering. *Materials science & engineering. C, Materials for biological applications* **2017**, *81*, 74-83.
157. Yang, L.; Korom, S.; Welti, M.; Hoerstrup, S. P.; Zünd, G.; Jung, F. J.; Neuenschwander, P.; Weder, W., Tissue engineered cartilage generated from human trachea using DegraPol® scaffold. *European journal of cardio-thoracic surgery* **2003**, *24* (2), 201-207.
158. Best, C. A.; Pepper, V. K.; Ohst, D.; Bodnyk, K.; Heuer, E.; Onwuka, E. A.; King, N.; Strouse, R.; Grischkan, J.; Breuer, C. K.; Johnson, J.; Chiang, T., Designing a tissue-engineered tracheal scaffold for preclinical evaluation. *International journal of pediatric otorhinolaryngology* **2018**, *104*, 155-160.
159. Kang, Y.; Wang, C.; Qiao, Y.; Gu, J.; Zhang, H.; Peijs, T.; Kong, J.; Zhang, G.; Shi, X., Tissue-Engineered Trachea Consisting of Electrospun Patterned sc-PLA/GO-g-IL Fibrous Membranes

- with Antibacterial Property and 3D-Printed Skeletons with Elasticity. *Biomacromolecules* **2019**, *20* (4), 1765-1776.
160. Moers-Carpi, M. M.; Sherwood, S., Polycaprolactone for the correction of nasolabial folds: a 24-month, prospective, randomized, controlled clinical trial. *Dermatologic Surgery* **2013**, *39* (3pt1), 457-463.
 161. Holländer, J.; Genina, N.; Jukarainen, H.; Khajeheian, M.; Rosling, A.; Mäkilä, E.; Sandler, N., Three-dimensional printed PCL-based implantable prototypes of medical devices for controlled drug delivery. *Journal of pharmaceutical sciences* **2016**, *105* (9), 2665-2676.
 162. Malikmammadov, E.; Tanir, T. E.; Kiziltay, A.; Hasirci, V.; Hasirci, N., PCL and PCL-based materials in biomedical applications. *Journal of Biomaterials science, Polymer edition* **2018**, *29* (7-9), 863-893.
 163. Maquet, V.; Pagnouille, C.; Evrard, B.; Jerome, R.; Foidart, J.-M.; Frankenne, F., Active Substance Delivery System Comprising A Hydrogel Atrix And Microcarriers. Google Patents: 2008.
 164. Young, B. M.; Shankar, K.; Allen, B. P.; Pouliot, R. A.; Schneck, M. B.; Mikhael, N. S.; Heise, R. L., Electrospun decellularized lung matrix scaffold for airway smooth muscle culture. *ACS Biomaterials Science & Engineering* **2017**, *3* (12), 3480-3492.
 165. Hamaji, M.; Kojima, F.; Koyasu, S.; Tsuruyama, T.; Komatsu, T.; Ikuno, T.; Date, H.; Nakamura, T., Development of a composite and vascularized tracheal scaffold in the omentum for in situ tissue engineering: a canine model. *Interactive cardiovascular and thoracic surgery* **2014**, *19* (3), 357-362.
 166. Lin, C.-H.; Su, J.-M.; Hsu, S.-H., Evaluation of type II collagen scaffolds reinforced by poly (ϵ -caprolactone) as tissue-engineered trachea. *Tissue Engineering Part C: Methods* **2008**, *14* (1), 69-77.
 167. Kim, M. S.; Kim, G., Three-dimensional electrospun polycaprolactone (PCL)/alginate hybrid composite scaffolds. *Carbohydrate polymers* **2014**, *114*, 213-221.
 168. Chen, G.; Sato, T.; Ushida, T.; Hirochika, R.; Shirasaki, Y.; Ochiai, N.; Tateishi, T., The use of a novel PLGA fiber/collagen composite web as a scaffold for engineering of articular cartilage tissue with adjustable thickness. *Journal of Biomedical Materials Research Part A: An Official Journal of the Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials* **2003**, *67* (4), 1170-1180.
 169. Bryksin, A. V.; Brown, A. C.; Baksh, M. M.; Finn, M. G.; Barker, T. H., Learning from nature—novel synthetic biology approaches for biomaterial design. *Acta biomaterialia* **2014**, *10* (4), 1761-1769.
 170. de Oliveira, F. C. S.; do Amaral, R. J. F. C.; Dos Santos, L. E. C.; Cummins, C.; Morris, M. M.; Kearney, C. J.; Heise, A., Versatility of unsaturated polyesters from electrospun macrolactones: RGD immobilization to increase cell attachment. *Journal of Biomedical Materials Research Part A* **2021**.
 171. Ates, Z.; Thornton, P. D.; Heise, A., Side-chain functionalisation of unsaturated polyesters from ring-opening polymerisation of macrolactones by thiol–ene click chemistry. *Polymer Chemistry* **2011**, *2* (2), 309-312.
 172. van der Meulen, I.; de Geus, M.; Antheunis, H.; Deumens, R.; Joosten, E. A. J.; Koning, C. E.; Heise, A., Polymers from functional macrolactones as potential biomaterials: enzymatic ring opening polymerization, biodegradation, and biocompatibility. *Biomacromolecules* **2008**, *9* (12), 3404-3410.
 173. de Oliveira, F. C. S.; Olvera, D.; Sawkins, M. J.; Cryan, S.-A.; Kimmins, S. D.; da Silva, T. E.; Kelly, D. J.; Duffy, G. P.; Kearney, C.; Heise, A., Direct UV-Triggered thiol–ene cross-linking of electrospun polyester fibers from unsaturated poly (macrolactone) s and their drug loading by solvent swelling. *Biomacromolecules* **2017**, *18* (12), 4292-4298.
 174. Rey, D. F. V.; St-Pierre, J.-P., Fabrication techniques of tissue engineering scaffolds. In *Handbook of Tissue Engineering Scaffolds: Volume One*, Elsevier: 2019; pp 109-125.
 175. Hsieh, C.-T.; Liao, C.-Y.; Dai, N.-T.; Tseng, C.-S.; Yen, B. L.; Hsu, S.-h., 3D printing of tubular scaffolds with elasticity and complex structure from multiple waterborne polyurethanes for tracheal tissue engineering. *Applied Materials Today* **2018**, *12*, 330-341.
 176. Wu, T.; Zheng, H.; Chen, J.; Wang, Y.; Sun, B.; Morsi, Y.; El-Hamshary, H.; Al-Deyab, S. S.; Chen, C.; Mo, X., Application of a bilayer tubular scaffold based on electrospun poly (l-lactide-co-caprolactone)/collagen fibers and yarns for tracheal tissue engineering. *Journal of Materials Chemistry B* **2017**, *5* (1), 139-150.
 177. Lee, D.; Wu, G.-Y., Parameters affecting the mechanical properties of three-dimensional (3D) printed carbon fiber-reinforced polylactide composites. *Polymers* **2020**, *12* (11), 2456.
 178. Murphy, C. M.; Haugh, M. G.; O'Brien, F. J., The effect of mean pore size on cell attachment, proliferation and migration in collagen–glycosaminoglycan scaffolds for bone tissue engineering. *Biomaterials* **2010**, *31* (3), 461-466.

179. Tamay, D. G.; Dursun Usal, T.; Alagoz, A. S.; Yucel, D.; Hasirci, N.; Hasirci, V., 3D and 4D printing of polymers for tissue engineering applications. *Frontiers in bioengineering and biotechnology* **2019**, 7, 164.
180. Lee, S. J.; Choi, J. S.; Eom, M. R.; Jo, H. H.; Kwon, I. K.; Kwon, S. K.; Park, S. A., Dexamethasone loaded bilayered 3D tubular scaffold reduces restenosis at the anastomotic site of tracheal replacement: in vitro and in vivo assessments. *Nanoscale* **2020**, 12 (8), 4846-4858.
181. Xu, Y.; Li, D.; Yin, Z.; He, A.; Lin, M.; Jiang, G.; Song, X.; Hu, X.; Liu, Y.; Wang, J., Tissue-engineered trachea regeneration using decellularized trachea matrix treated with laser micropore technique. *Acta biomaterialia* **2017**, 58, 113-121.
182. Zang, M.; Zhang, Q.; Davis, G.; Huang, G.; Jaffari, M.; Ríos, C. N.; Gupta, V.; Yu, P.; Mathur, A. B., Perichondrium directed cartilage formation in silk fibroin and chitosan blend scaffolds for tracheal transplantation. *Acta biomaterialia* **2011**, 7 (9), 3422-31.
183. Badylak, S. F.; Tullius, R.; Kokini, K.; Shelbourne, K. D.; Klootwyk, T.; Voytik, S. L.; Kraine, M. R.; Simmons, C., The use of xenogeneic small intestinal submucosa as a biomaterial for Achilles tendon repair in a dog model. *Journal of biomedical materials research* **1995**, 29 (8), 977-985.
184. Shojaie, S.; Lee, J.; Wang, J.; Ackerley, C.; Post, M., Generation of ESC-derived mouse airway epithelial cells using decellularized lung scaffolds. *Journal of visualized experiments: JoVE* **2016**, (111).
185. Price, A. P.; England, K. A.; Matson, A. M.; Blazar, B. R.; Panoskaltsis-Mortari, A., Development of a decellularized lung bioreactor system for bioengineering the lung: the matrix reloaded. *Tissue engineering Part A* **2010**, 16 (8), 2581-2591.
186. Ohno, M.; Fuchimoto, Y.; Hsu, H. C.; Higuchi, M.; Komura, M.; Yamaoka, T.; Umezawa, A.; Enosawa, S.; Kuroda, T., Airway reconstruction using decellularized tracheal allografts in a porcine model. *Pediatric surgery international* **2017**, 33 (10), 1065-1071.
187. Hamilton, N.; Bullock, A. J.; Macneil, S.; Janes, S. M.; Birchall, M., Tissue engineering airway mucosa: a systematic review. *The Laryngoscope* **2014**, 124 (4), 961-8.
188. Rehmani, S. S.; Al-Ayoubi, A. M.; Ayub, A.; Barsky, M.; Lewis, E.; Flores, R.; Lebovics, R.; Bhora, F. Y., Three-dimensional-printed bioengineered tracheal grafts: preclinical results and potential for human use. *The Annals of Thoracic Surgery* **2017**, 104 (3), 998-1004.
189. Den Hondt, M.; Vanaudenaerde, B. M.; Verbeken, E. K.; Vranckx, J. J., Epithelial grafting of a decellularized whole-tracheal segment: an in vivo experimental model. *Interactive cardiovascular and thoracic surgery* **2018**, 26 (5), 753-760.
190. Haag, J.; Baiguera, S.; Jungebluth, P.; Barale, D.; Del Gaudio, C.; Castiglione, F.; Bianco, A.; Comin, C. E.; Ribatti, D.; Macchiarini, P., Biomechanical and angiogenic properties of tissue-engineered rat trachea using genipin cross-linked decellularized tissue. *Biomaterials* **2012**, 33 (3), 780-789.
191. Crowley, C.; Birchall, M.; Seifalian, A. M., Trachea transplantation: from laboratory to patient. *Journal of tissue engineering and regenerative medicine* **2015**, 9 (4), 357-367.
192. Racine, L.; Texier, I.; Auzély-Velty, R., Chitosan-based hydrogels: recent design concepts to tailor properties and functions. *Polymer International* **2017**, 66 (7), 981-998.
193. Catoira, M. C.; Fusaro, L.; Di Francesco, D.; Ramella, M.; Boccafroschi, F., Overview of natural hydrogels for regenerative medicine applications. *Journal of Materials Science: Materials in Medicine* **2019**, 30 (10), 1-10.
194. Tam, R. Y.; Yockell-Lelièvre, J.; Smith, L. J.; Julian, L. M.; Baker, A. E. G.; Choey, C.; Hasim, M. S.; Dimitroulakos, J.; Stanford, W. L.; Shoichet, M. S., Rationally Designed 3D Hydrogels Model Invasive Lung Diseases Enabling High-Content Drug Screening. *Advanced Materials* **2019**, 31 (7), 1806214.
195. Falcones, B.; Sanz-Fraile, H.; Marhuenda, E.; Mendizábal, I.; Cabrera-Aguilera, I.; Malandain, N.; Uriarte, J. J.; Almendros, I.; Navajas, D.; Weiss, D. J., Bioprintable Lung Extracellular Matrix Hydrogel Scaffolds for 3D Culture of Mesenchymal Stromal Cells. *Polymers* **2021**, 13 (14), 2350.
196. Almendros, I.; Otero, J.; Falcones, B.; Marhuenda, E.; Navajas, D.; Farre, R., Lung extracellular matrix hydrogel for 3D bioprinting of lung mesenchymal stem cells. *Eur Respiratory Soc*: **2019**.
197. Xu, Y.; Wang, Z.; Hua, Y.; Zhu, X.; Wang, Y.; Duan, L.; Zhu, L.; Jiang, G.; Xia, H.; She, Y., Photocrosslinked natural hydrogel composed of hyaluronic acid and gelatin enhances cartilage regeneration of decellularized trachea matrix. *Materials Science and Engineering: C* **2021**, 120, 111628.
198. Chen, H.; Wang, H.; Li, B.; Feng, B.; He, X.; Fu, W.; Yuan, H.; Xu, Z., Enhanced chondrogenic differentiation of human mesenchymal stem cells on citric acid-modified chitosan hydrogel for tracheal cartilage regeneration applications. *RSC advances* **2018**, 8 (30), 16910-16917.

199. Kim, S. H.; Seo, Y. B.; Yeon, Y. K.; Lee, Y. J.; Park, H. S.; Sultan, M. T.; Lee, J. M.; Lee, J. S.; Lee, O. J.; Hong, H., 4D-bioprinted silk hydrogels for tissue engineering. *Biomaterials* **2020**, *260*, 120281.
200. Najaf Zadeh, H.; Huber, T.; Nock, V.; Fee, C.; Clucas, D., Complex Geometry Cellulose Hydrogels Using a Direct Casting Method. *Bioengineering* **2020**, *7* (2), 58.
201. Naito, H.; Tojo, T.; Kimura, M.; Dohi, Y.; Zimmermann, W.-H.; Eschenhagen, T.; Taniguchi, S., Engineering bioartificial tracheal tissue using hybrid fibroblast-mesenchymal stem cell cultures in collagen hydrogels. *Interactive cardiovascular and thoracic surgery* **2011**, *12* (2), 156-161.
202. Kreimendahl, F.; Ossenbrink, S.; Köpf, M.; Westhofen, M.; Schmitz-Rode, T.; Fischer, H.; Jockenhoevel, S.; Thiebes, A. L., Combination of vascularization and cilia formation for three-dimensional airway tissue engineering. *Journal of biomedical materials research. Part A* **2019**, *107* (9), 2053-2062.
203. Jun, I.; Han, H. S.; Edwards, J. R.; Jeon, H., Electrospun Fibrous Scaffolds for Tissue Engineering: Viewpoints on Architecture and Fabrication. *International journal of molecular sciences* **2018**, *19* (3).
204. Morris, G. E.; Bridge, J. C.; Eltboli, O. M. I.; Lewis, M. P.; Knox, A. J.; Aylott, J. W.; Brightling, C. E.; Ghaemmaghami, A. M.; Rose, F. R. A. J., Human airway smooth muscle maintain in situ cell orientation and phenotype when cultured on aligned electrospun scaffolds. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **2014**, *307* (1), L38-L47.
205. Morris, G. E.; Bridge, J. C.; Brace, L. A.; Knox, A. J.; Aylott, J. W.; Brightling, C. E.; Ghaemmaghami, A. M.; Rose, F. R. A. J., A novel electrospun biphasic scaffold provides optimal three-dimensional topography for in vitro co-culture of airway epithelial and fibroblast cells. *Biofabrication* **2014**, *6* (3), 035014.
206. Pepper, V.; Best, C. A.; Buckley, K.; Schwartz, C.; Onwuka, E.; King, N.; White, A.; Dharmadhikari, S.; Reynolds, S. D.; Johnson, J. J. O. H.; Surgery, N., Factors Influencing Poor Outcomes in Synthetic Tissue-Engineered Tracheal Replacement. **2019**, *161* (3), 458-467.
207. Townsend, J. M.; Ott, L. M.; Salash, J. R.; Fung, K. M.; Easley, J. T.; Seim, H. B., 3rd; Johnson, J. K.; Weatherly, R. A.; Detamore, M. S., Reinforced Electrospun Polycaprolactone Nanofibers for Tracheal Repair in an In Vivo Ovine Model. *Tissue engineering. Part A* **2018**, *24* (17-18), 1301-1308.
208. Dharmadhikari, S.; Best, C. A.; King, N.; Henderson, M.; Johnson, J.; Breuer, C. K.; Chiang, T., Mouse model of tracheal replacement with electrospun nanofiber scaffolds. *Annals of Otology, Rhinology & Laryngology* **2019**, *128* (5), 391-400.
209. Pensa, N. W.; Curry, A. S.; Bonvallet, P. P.; Bellis, N. F.; Rettig, K. M.; Reddy, M. S.; Eberhardt, A. W.; Bellis, S. L., 3D printed mesh reinforcements enhance the mechanical properties of electrospun scaffolds. *Biomaterials research* **2019**, *23* (1), 1-7.
210. Deslauriers, J.; Aubrée, N.; Shamji, F. M., Experiences with Prosthetic Airway Replacement. *Thoracic surgery clinics* **2018**, *28* (3), 377-384.
211. Les, A. S.; Ohye, R. G.; Filbrun, A. G.; Ghadimi Mahani, M.; Flanagan, C. L.; Daniels, R. C.; Kidwell, K. M.; Zopf, D. A.; Hollister, S. J.; Green, G. E., 3D-printed, externally-implanted, bioresorbable airway splints for severe tracheobronchomalacia. *The Laryngoscope* **2019**, *129* (8), 1763-1771.
212. Wu, L.; Magaz, A.; Huo, S.; Darbyshire, A.; Loizidou, M.; Emberton, M.; Birchall, M.; Song, W., Human airway-like multilayered tissue on 3D-TIPS printed thermoresponsive elastomer/collagen hybrid scaffolds. *Acta biomaterialia* **2020**, *113*, 177-195.
213. Park, J. Y.; Ryu, H.; Lee, B.; Ha, D.-H.; Ahn, M.; Kim, S.; Kim, J. Y.; Jeon, N. L.; Cho, D.-W., Development of a functional airway-on-a-chip by 3D cell printing. *Biofabrication* **2018**, *11* (1), 015002.
214. Morrison, R. J.; Hollister, S. J.; Niedner, M. F.; Mahani, M. G.; Park, A. H.; Mehta, D. K.; Ohye, R. G.; Green, G. E., Mitigation of tracheobronchomalacia with 3D-printed personalized medical devices in pediatric patients. *Science translational medicine* **2015**, *7* (285), 285ra64.
215. Xia, D.; Jin, D.; Wang, Q.; Gao, M.; Zhang, J.; Zhang, H.; Bai, J.; Feng, B.; Chen, M.; Huang, Y.; Zhong, Y.; Witman, N.; Wang, W.; Xu, Z.; Zhang, H.; Yin, M.; Fu, W., Tissue-engineered trachea from a 3D-printed scaffold enhances whole-segment tracheal repair in a goat model. *Journal of tissue engineering and regenerative medicine* **2019**, *13* (4), 694-703.
216. An, J.; Chua, C. K., An engineering perspective on 3D printed personalized scaffolds for tracheal suspension technique. *Journal of thoracic disease* **2016**, *8* (12), E1723.
217. Gao, B.; Jing, H.; Gao, M.; Wang, S.; Fu, W.; Zhang, X.; He, X.; Zheng, J., Long-segmental tracheal reconstruction in rabbits with pedicled Tissue-engineered trachea based on a 3D-printed scaffold. *Acta biomaterialia* **2019**, *97*, 177-186.

218. Atala, A.; Forgacs, G., Three-Dimensional Bioprinting in Regenerative Medicine: Reality, Hype, and Future. *Stem cells translational medicine* **2019**, *8* (8), 744-745.
219. Kaye, R.; Goldstein, T.; Grande, D. A.; Zeltsman, D.; Smith, L. P., A 3-dimensional bioprinted tracheal segment implant pilot study: Rabbit tracheal resection with graft implantation. *International journal of pediatric otorhinolaryngology* **2019**, *117*, 175-178.
220. Bae, S. W.; Lee, K. W.; Park, J. H.; Lee, J.; Jung, C. R.; Yu, J.; Kim, H. Y.; Kim, D. H., 3D Bioprinted Artificial Trachea with Epithelial Cells and Chondrogenic-Differentiated Bone Marrow-Derived Mesenchymal Stem Cells. *International journal of molecular sciences* **2018**, *19* (6).
221. She, Y.; Fan, Z.; Wang, L.; Li, Y.; Sun, W.; Tang, H.; Zhang, L.; Wu, L.; Zheng, H.; Chen, C., 3D Printed Biomimetic PCL Scaffold as framework interspersed with collagen for long segment tracheal replacement. *Frontiers in cell and developmental biology* **2021**, *9*, 33.
222. Frejo, L.; Grande, D. A., 3D-bioprinted tracheal reconstruction: an overview. *Bioelectronic medicine* **2019**, *5* (1), 1-7.
223. Selo, M. A.; Sake, J. A.; Kim, K.-J.; Ehrhardt, C., In vitro and ex vivo models in inhalation biopharmaceutical research—advances, challenges and future perspectives. *Advanced Drug Delivery Reviews* **2021**, 113862.
224. Miller, A. J.; Spence, J. R., In vitro models to study human lung development, disease and homeostasis. *Physiology* **2017**, *32* (3), 246-260.
225. Strong, P.; Ito, K.; Murray, J.; Rapeport, G., Current approaches to the discovery of novel inhaled medicines. *Drug discovery today* **2018**, *23* (10), 1705-1717.
226. Cidem, A.; Bradbury, P.; Traini, D.; Ong, H. X., Modifying and Integrating in vitro and ex vivo Respiratory Models for Inhalation Drug Screening. *Frontiers in bioengineering and biotechnology* **2020**, *8*, 1256.
227. Gribaldo, L.; Hurley, K.; Hiemstra, P.; Greene, C., Increased focus on non-animal models for COVID-19 and non-COVID lung research. *European Respiratory Journal* **2021**, *57* (1).
228. Marrazzo, P.; Maccari, S.; Taddei, A.; Bevan, L.; Telford, J.; Soriani, M.; Pezzicoli, A., 3D Reconstruction of the Human Airway Mucosa In Vitro as an Experimental Model to Study NTHi Infections. *PloS one* **2016**, *11* (4), e0153985.
229. Hasan, S.; Sebo, P.; Osicka, R., A guide to polarized airway epithelial models for studies of host–pathogen interactions. *The FEBS journal* **2018**, *285* (23), 4343-4358.
230. Grainger, C. I.; Greenwell, L. L.; Lockley, D. J.; Martin, G. P.; Forbes, B., Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharmaceutical research* **2006**, *23* (7), 1482-1490.
231. Smithmyer, M. E.; Cassel, S. E.; Kloxin, A. M., Bridging 2D and 3D culture: probing impact of extracellular environment on fibroblast activation in layered hydrogels. *AIChE Journal* **2019**, *65* (12), e16837.
232. Yonker, L. M.; Mou, H.; Chu, K. K.; Pazos, M. A.; Leung, H.; Cui, D.; Ryu, J.; Hibbler, R. M.; Eaton, A. D.; Ford, T. N., Development of a primary human co-culture model of inflamed airway mucosa. *Scientific reports* **2017**, *7* (1), 1-12.
233. Bissell, M. J.; Radisky, D. C.; Rizki, A.; Weaver, V. M.; Petersen, O. W., The organizing principle: microenvironmental influences in the normal and malignant breast. *Differentiation* **2002**, *70* (9-10), 537-546.
234. Pampaloni, F.; Reynaud, E. G.; Stelzer, E. H. K., The third dimension bridges the gap between cell culture and live tissue. *Nature reviews Molecular cell biology* **2007**, *8* (10), 839-845.
235. Kook, Y.-M.; Jeong, Y.; Lee, K.; Koh, W.-G., Design of biomimetic cellular scaffolds for co-culture system and their application. *Journal of tissue engineering* **2017**, *8*, 2041731417724640.
236. Roskelley, C. D.; Desprez, P. Y.; Bissell, M. J., Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proceedings of the National Academy of Sciences* **1994**, *91* (26), 12378-12382.
237. Griffith, L. G.; Swartz, M. A., Capturing complex 3D tissue physiology in vitro. *Nature reviews Molecular cell biology* **2006**, *7* (3), 211-224.
238. Bachrach, N. M.; Valhmu, W. B.; Stazzone, E.; Ratcliffe, A.; Lai, W. M.; Mow, V. C., Changes in proteoglycan synthesis of chondrocytes in articular cartilage are associated with the time-dependent changes in their mechanical environment. *J Biomech* **1995**, *28* (12), 1561-1569.
239. Frantz, C.; Stewart, K. M.; Weaver, V. M., The extracellular matrix at a glance. *Journal of cell science* **2010**, *123* (24), 4195-4200.
240. Miller, C.; George, S.; Niklason, L., Developing a tissue-engineered model of the human bronchiole. *Journal of tissue engineering and regenerative medicine* **2010**, *4* (8), 619-627.

241. Choe, M. M.; Sporn, P. H. S.; Swartz, M. A., Extracellular matrix remodeling by dynamic strain in a three-dimensional tissue-engineered human airway wall model. *American journal of respiratory cell and molecular biology* **2006**, 35 (3), 306-313.
242. Rothen-Rutishauser, B. M.; Kiama, S. G.; Gehr, P., A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. *American journal of respiratory cell and molecular biology* **2005**, 32 (4), 281-289.
243. Ishikawa, S.; Ishimori, K.; Ito, S., A 3D epithelial–mesenchymal co-culture model of human bronchial tissue recapitulates multiple features of airway tissue remodeling by TGF- β 1 treatment. *Respiratory research* **2017**, 18 (1), 1-11.
244. Arumugasaamy, N.; Navarro, J.; Leach, J. K.; Kim, P. C. W.; Fisher, J. P., In vitro models for studying transport across epithelial tissue barriers. *Annals of biomedical engineering* **2019**, 47 (1), 1-21.
245. Chandorkar, P.; Posch, W.; Zaderer, V.; Blatzer, M.; Steger, M.; Ammann, C. G.; Binder, U.; Hermann, M.; Hörtnagl, P.; Lass-Flörl, C., Fast-track development of an in vitro 3D lung/immune cell model to study Aspergillus infections. *Scientific reports* **2017**, 7 (1), 1-13.
246. Wrzesinski, K.; J Fey, S., From 2D to 3D-a new dimension for modelling the effect of natural products on human tissue. *Current pharmaceutical design* **2015**, 21 (38), 5605-5616.
247. Pasman, T.; Baptista, D.; van Riet, S.; Truckenmüller, R. K.; Hiemstra, P. S.; Rottier, R. J.; Hamelmann, N. M.; Paulusse, J. M. J.; Stamatialis, D.; Poot, A. A., Development of an In Vitro Airway Epithelial–Endothelial Cell Culture Model on a Flexible Porous Poly (Trimethylene Carbonate) Membrane Based on Calu-3 Airway Epithelial Cells and Lung Microvascular Endothelial Cells. *Membranes* **2021**, 11 (3), 197.
248. Shanks, N.; Greek, R.; Greek, J., Are animal models predictive for humans? *Philosophy, ethics, and humanities in medicine* **2009**, 4 (1), 1-20.
249. Nahar, K.; Gupta, N.; Gauvin, R.; Absar, S.; Patel, B.; Gupta, V.; Khademhosseini, A.; Ahsan, F., In vitro, in vivo and ex vivo models for studying particle deposition and drug absorption of inhaled pharmaceuticals. *European journal of pharmaceutical sciences* **2013**, 49 (5), 805-818.
250. Barkauskas, C. E.; Chung, M.-I.; Fioret, B.; Gao, X.; Katsura, H.; Hogan, B. L. M., Lung organoids: current uses and future promise. *Development* **2017**, 144 (6), 986-997.
251. Shin, T. H.; Kim, M.; Sung, C. O.; Jang, S. J.; Jeong, G. S., A one-stop microfluidic-based lung cancer organoid culture platform for testing drug sensitivity. *Lab on a Chip* **2019**, 19 (17), 2854-2865.
252. Nam, K.-H.; Smith, A. S. T.; Lone, S.; Kwon, S.; Kim, D.-H., Biomimetic 3D tissue models for advanced high-throughput drug screening. *Journal of laboratory automation* **2015**, 20 (3), 201-215.
253. Kabadi, P. K.; Rodd, A. L.; Simmons, A. E.; Messier, N. J.; Hurt, R. H.; Kane, A. B., A novel human 3D lung microtissue model for nanoparticle-induced cell-matrix alterations. *Particle and fibre toxicology* **2019**, 16 (1), 15.
254. Gómez-Caro Andrés, A.; Moradiellos Díez, F. J.; Ausín Herrero, P.; Díaz-Hellín Gude, V.; Larrú Cabrero, E.; de Miguel Porch, E.; Martín De Nicolás, J. L., Successful conservative management in iatrogenic tracheobronchial injury. *Ann Thorac Surg* **2005**, 79 (6), 1872-8.
255. Prokakis, C.; Koletsis, E. N.; Dedeilias, P.; Fligou, F.; Filos, K.; Dougenis, D., Airway trauma: a review on epidemiology, mechanisms of injury, diagnosis and treatment. *Journal of cardiothoracic surgery* **2014**, 9, 117.
256. Madariaga, M. L. L.; Gaissert, H. A., Overview of malignant tracheal tumors. *Annals of cardiothoracic surgery* **2018**, 7 (2), 244-254.
257. Nassar, N.; Leoncini, E.; Amar, E.; Arteaga-Vázquez, J.; Bakker, M. K.; Bower, C.; Canfield, M. A.; Castilla, E. E.; Cocchi, G.; Correa, A.; Csáky-Szunyogh, M.; Feldkamp, M. L.; Khoshnood, B.; Landau, D.; Lelong, N.; López-Camelo, J. S.; Lowry, R. B.; McDonnell, R.; Merlob, P.; Métneki, J.; Morgan, M.; Mutchinick, O. M.; Palmer, M. N.; Rissmann, A.; Siffel, C.; Sipek, A.; Szabova, E.; Tucker, D.; Mastroiacovo, P., Prevalence of esophageal atresia among 18 international birth defects surveillance programs. *Birth defects research. Part A, Clinical and molecular teratology* **2012**, 94 (11), 893-9.
258. Ergun, S.; Tewfik, T.; Daniel, S., Tracheal agenesis: A rare but fatal congenital anomaly. *McGill journal of medicine : MJM : an international forum for the advancement of medical sciences by students* **2011**, 13 (1), 10.
259. Shaw-Smith, C., Oesophageal atresia, tracheo-oesophageal fistula, and the VACTERL association: review of genetics and epidemiology. *Journal of medical genetics* **2006**, 43 (7), 545-54.
260. Esteller-Moré, E.; Ibañez, J.; Matión, E.; Ademà, J. M.; Nolla, M.; Quer, I. M., Prognostic factors in laryngotracheal injury following intubation and/or tracheotomy in ICU patients. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-*

Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery **2005**, 262 (11), 880-3.

261. Sarper, A.; Ayten, A.; Eser, I.; Ozbudak, O.; Demircan, A., Tracheal stenosis after tracheostomy or intubation: review with special regard to cause and management. *Texas Heart Institute journal* **2005**, 32 (2), 154-8.

262. Mattioli, F.; Marchioni, A.; Andreani, A.; Cappiello, G.; Fermi, M.; Presutti, L., Post-intubation tracheal stenosis in COVID-19 patients. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) : affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery* **2020**, 1-2.

263. Cheng, F.-Y.; Joshi, H.; Tandon, P.; Freeman, R.; Reich, D. L.; Mazumdar, M.; Kohli-Seth, R.; Levin, M. A.; Timsina, P.; Kia, A., Using machine learning to predict ICU transfer in hospitalized COVID-19 patients. *Journal of clinical medicine* **2020**, 9 (6), 1668.

264. Grillo, H. C., Tracheal replacement: a critical review. *The Annals of Thoracic Surgery* **2002**, 73 (6), 1995-2004.

265. Grillo, H. C., Slide tracheoplasty for long-segment congenital tracheal stenosis. *Ann Thorac Surg* **1994**, 58 (3), 613-9 discussion 619-21.

266. Den Hondt, M.; Vanaudenaerde, B.; Verbeken, E.; Vranckx, J. J. J. P.; Surgery, R., Requirements for successful trachea transplantation: a study in the rabbit model. **2018**, 141 (6), 845e-856e.

267. Walles, T.; Giere, B.; Hofmann, M.; Schanz, J.; Hofmann, F.; Mertsching, H.; Macchiarini, P. J. T. J. o. t.; surgery, c., Experimental generation of a tissue-engineered functional and vascularized trachea. **2004**, 128 (6), 900-906.

268. Graziano, J. L.; Spinazzola, A.; Neville, W. E., Prosthetic replacement of the tracheal carina. *Ann Thorac Surg* **1967**, 4 (1), 1-11.

269. Neville, W. E.; Bolanowski, J. P.; Kotia, G. G., Clinical experience with the silicone tracheal prosthesis. *J Thorac Cardiovasc Surg* **1990**, 99 (4), 604-12; discussion 612-3.

270. Beall, A. C., Jr.; Harrington, O. B.; Greenberg, S. D.; Morris, G. C., Jr.; Usher, F. C., Tracheal replacement with heavy Marlex mesh. Circumferential replacement of the cervical trachea. *Archives of surgery (Chicago, Ill. : 1920)* **1962**, 84, 390-6.

271. Clagett, O. T.; Grindlay, J. H.; Moersch, H. J., Resection of the trachea; an experimental study and a report of a case. *Archives of surgery (Chicago, Ill. : 1920)* **1948**, 57 (2), 253-66.

272. Longmire, W. P., Jr., The repair of large defects of the trachea. *The Annals of otology, rhinology, and laryngology* **1948**, 57 (3), 875-83.

273. Gebauer, P. W., Experiences with surgical reconstruction of the trachea. *American review of tuberculosis* **1950**, 62 (2), 176-89.

274. Bucher, R. M.; Burnett, W. E.; Rosemond, G. P., Experimental reconstruction of tracheal and bronchial defects with stainless steel wire mesh. *The Journal of thoracic surgery* **1951**, 21 (6), 572-83.

275. Pearson, F. G.; Henderson, R. D.; Gross, A. E.; Ginsberg, R. J.; Stone, R. M., The reconstruction of circumferential tracheal defects with a porous prosthesis. An experimental and clinical study using heavy Marlex mesh. *J Thorac Cardiovasc Surg* **1968**, 55 (5), 605-16.

276. Montgomery, W. W., Silicone tracheal T-tube. *The Annals of otology, rhinology, and laryngology* **1974**, 83 (1), 71-5.

277. Husain, S. A.; Finch, D.; Ahmed, M.; Morgan, A.; Hetzel, M. R., Long-Term Follow-Up of Ultraflex Metallic Stents in Benign and Malignant Central Airway Obstruction. *The Annals of Thoracic Surgery* **2007**, 83 (4), 1251-1256.

278. Shepherd, R. W.; Lamb, C.; Beamis, J. F., CLINICAL EXPERIENCE OF THE POLYFLEX[™] STENT IN BENIGN AND MALIGNANT CENTRAL AIRWAY OBSTRUCTION. *CHEST* **2006**, 130 (4), 110S.

279. Folch, E.; Keyes, C. J. A. o. C. S., Airway stents. **2018**, 7 (2), 273-283.

280. Terra, R. M.; Minamoto, H.; Tedde, M. L.; Almeida, J. L. J. d.; Jatene, F. B., Self-expanding stent made of polyester mesh with silicon coating (Polyflex®) in the treatment of inoperable tracheal stenoses. *Jornal Brasileiro de Pneumologia* **2007**, 33, 241-247.

281. Guibert, N.; Saka, H.; Dutau, H., Airway stenting: Technological advancements and its role in interventional pulmonology. *Respirology (Carlton, Vic.)* **2020**, 25 (9), 953-962.

282. Dutau, H.; Toutblanc, B.; Lamb, C.; Seijo, L., Use of the Dumon Y-stent in the management of malignant disease involving the carina: a retrospective review of 86 patients. *Chest* **2004**, 126 (3), 951-958.

283. Freitag, L.; Tekolf, E.; Stamatis, C.; Greschuchna, D., Clinical evaluation of a new bifurcated dynamic airway stent: a 5-year experience with 135 patients. *The Thoracic and cardiovascular surgeon* **1997**, 45 (01), 6-12.

284. Seguin, A.; Radu, D.; Holder-Espinasse, M.; Bruneval, P.; Fialaire-Legendre, A.; Duterque-Coquillaud, M.; Carpentier, A.; Martinod, E., Tracheal replacement with cryopreserved, decellularized, or glutaraldehyde-treated aortic allografts. *Ann Thorac Surg* **2009**, *87* (3), 861-7.
285. Tsukada, H.; Ernst, A.; Gangadharan, S.; Ashiku, S.; Garland, R.; Litmanovich, D.; DeCamp, M., Tracheal replacement with a silicone-stented, fresh aortic allograft in sheep. *Ann Thorac Surg* **2010**, *89* (1), 253-8.
286. Wurtz, A.; Hysi, I., [Tracheal replacement with aortic allografts in humans. Experimental prospects]. *Revue des maladies respiratoires* **2012**, *29* (7), 941-4.
287. Azorin, J. F.; Bertin, F.; Martinod, E.; Laskar, M., Tracheal replacement with an aortic autograft. *European Journal of Cardio-Thoracic Surgery* **2006**, *29* (2), 261-263.
288. Martinod, E.; Radu, D. M.; Chouahnia, K.; Seguin, A.; Fialaire-Legendre, A.; Brillet, P. Y.; Destable, M. D.; Sebbane, G.; Beloucif, S.; Valeyre, D.; Baillard, C.; Carpentier, A., Human transplantation of a biologic airway substitute in conservative lung cancer surgery. *Ann Thorac Surg* **2011**, *91* (3), 837-42.
289. Wurtz, A.; Porte, H.; Conti, M.; Dusson, C.; Desbordes, J.; Copin, M. C.; Marquette, C. H., Surgical technique and results of tracheal and carinal replacement with aortic allografts for salivary gland-type carcinoma. *J Thorac Cardiovasc Surg* **2010**, *140* (2), 387-393.e2.
290. Martinod, E.; Chouahnia, K.; Radu, D. M.; Joudiou, P.; Uzunhan, Y.; Bensidhoum, M.; Santos Portela, A. M.; Guiraudet, P.; Peretti, M.; Destable, M. D.; Solis, A.; Benachi, S.; Fialaire-Legendre, A.; Rouard, H.; Collon, T.; Piquet, J.; Leroy, S.; Vénissac, N.; Santini, J.; Tresallet, C.; Dutau, H.; Sebbane, G.; Cohen, Y.; Beloucif, S.; d'Audiffret, A. C.; Petite, H.; Valeyre, D.; Carpentier, A.; Vicaut, E., Feasibility of Bioengineered Tracheal and Bronchial Reconstruction Using Stented Aortic Matrices. *Jama* **2018**, *319* (21), 2212-2222.
291. Rose, K. G.; Sesterhenn, K.; Wustrow, F., Tracheal allotransplantation in man. *Lancet (London, England)* **1979**, *1* (8113), 433.
292. Levashov Yu, N.; Yablonsky, P. K.; Cherny, S. M.; Orlov, S. V.; Shafirovsky, B. B.; Kuznetsov, I. M., One-stage allotransplantation of thoracic segment of the trachea in a patient with idiopathic fibrosing mediastinitis and marked tracheal stenosis. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **1993**, *7* (7), 383-6.
293. Udelsman, B. V.; Eaton, J.; Muniappan, A.; Morse, C. R.; Wright, C. D.; Mathisen, D. J., Repair of large airway defects with bioprosthetic materials. *J Thorac Cardiovasc Surg* **2016**, *152* (5), 1388-1397.
294. Delaere, P. R.; Vranckx, J. J.; Meulemans, J.; Vander Poorten, V.; Segers, K.; Van Raemdonck, D.; De Leyn, P.; Decaluwé, H.; Doooms, C.; Verleden, G., Learning curve in tracheal allotransplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **2012**, *12* (9), 2538-45.
295. Delaere, P. R.; Vranckx, J. J.; Den Hondt, M., Tracheal allograft after withdrawal of immunosuppressive therapy. *The New England journal of medicine* **2014**, *370* (16), 1568-70.
296. Delaere, P.; Van Raemdonck, D., Tracheal replacement. *Journal of thoracic disease* **2016**, *8* (Suppl 2), S186-96.
297. Delaere, P.; Van Raemdonck, D.; Vranckx, J., Tracheal transplantation. *Intensive care medicine* **2019**, *45* (3), 391-393.
298. Omori, K.; Nakamura, T.; Kanemaru, S.; Asato, R.; Yamashita, M.; Tanaka, S.; Magrufov, A.; Ito, J.; Shimizu, Y., Regenerative medicine of the trachea: the first human case. *The Annals of otology, rhinology, and laryngology* **2005**, *114* (6), 429-33.
299. Okumuş, A.; Çizmeçi, O.; Kabakas, F.; Kuvat, S. V.; Bilir, A.; Aydin, A., Circumferential trachea reconstruction with a prefabricated axial bio-synthetic flap: experimental study. *International journal of pediatric otorhinolaryngology* **2005**, *69* (3), 335-344.
300. Go, T.; Jungebluth, P.; Baiguero, S.; Asnaghi, A.; Martorell, J.; Ostertag, H.; Mantero, S.; Birchall, M.; Bader, A.; Macchiarini, P., Both epithelial cells and mesenchymal stem cell-derived chondrocytes contribute to the survival of tissue-engineered airway transplants in pigs. *The Journal of thoracic and cardiovascular surgery* **2010**, *139* (2), 437-443.
301. Vacanti, C. A.; Paige, K. T.; Kim, W. S.; Sakata, J.; Upton, J.; Vacanti, J. P., Experimental tracheal replacement using tissue-engineered cartilage. *Journal of pediatric surgery* **1994**, *29* (2), 201-205.
302. Lin, C.-H.; Hsu, S.-h.; Huang, C.-E.; Cheng, W.-T.; Su, J.-M., A scaffold-bioreactor system for a tissue-engineered trachea. *Biomaterials* **2009**, *30* (25), 4117-4126.
303. Kim, J.; Suh, S. W.; Shin, J. Y.; Kim, J. H.; Choi, Y. S.; Kim, H., Replacement of a tracheal defect with a tissue-engineered prosthesis: early results from animal experiments. *The Journal of thoracic and cardiovascular surgery* **2004**, *128* (1), 124-129.

304. Sakaguchi, Y.; Sato, T.; Muranishi, Y.; Yutaka, Y.; Komatsu, T.; Omori, K.; Nakamura, T.; Date, H., Development of a novel tissue-engineered nitinol frame artificial trachea with native-like physical characteristics. *The Journal of thoracic and cardiovascular surgery* **2018**, *156* (3), 1264-1272.
305. Kim, J. H.; Kong, W. H.; Kim, J. G.; Kim, H. J.; Seo, S. W., Possibility of skin epithelial cell transdifferentiation in tracheal reconstruction. *Artificial organs* **2011**, *35* (2), 122-130.
306. Nakamura, T.; Sato, T.; Araki, M.; Ichihara, S.; Nakada, A.; Yoshitani, M.; Itoi, S.-i.; Yamashita, M.; Kanemaru, S.-i.; Omori, K., In situ tissue engineering for tracheal reconstruction using a luminal remodeling type of artificial trachea. *The Journal of thoracic and cardiovascular surgery* **2009**, *138* (4), 811-819.
307. Abbott, A., Medical Nobel prize committee deals with surgical scandal. *Nature News* **2016**, 537 (7620), 289.
308. Else, H., Scandal-weary Swedish government takes over research-fraud investigations. *Nature* **2019**, *571* (7764), 158-159.
309. Omori, K.; Tada, Y.; Suzuki, T.; Nomoto, Y.; Matsuzuka, T.; Kobayashi, K.; Nakamura, T.; Kanemaru, S.; Yamashita, M.; Asato, R., Clinical application of in situ tissue engineering using a scaffolding technique for reconstruction of the larynx and trachea. *The Annals of otology, rhinology, and laryngology* **2008**, *117* (9), 673-8.
310. Kanemaru, S.; Hirano, S.; Umeda, H.; Yamashita, M.; Suehiro, A.; Nakamura, T.; Maetani, T.; Omori, K.; Ito, J., A tissue-engineering approach for stenosis of the trachea and/or cricoid. *Acta otolaryngologica. Supplementum* **2010**, (563), 79-83.
311. Delaere, P.; Vranckx, J.; Verleden, G.; De Leyn, P.; Van Raemdonck, D., Tracheal Allotransplantation after Withdrawal of Immunosuppressive Therapy. **2010**, *362* (2), 138-145.
312. Elliott, M. J.; De Coppi, P.; Spegginorin, S.; Roebuck, D.; Butler, C. R.; Samuel, E.; Crowley, C.; McLaren, C.; Fierens, A.; Vondrys, D.; Cochrane, L.; Jephson, C.; Janes, S.; Beaumont, N. J.; Cogan, T.; Bader, A.; Seifalian, A. M.; Hsuan, J. J.; Lowdell, M. W.; Birchall, M. A., Stem-cell-based, tissue engineered tracheal replacement in a child: a 2-year follow-up study. *Lancet (London, England)* **2012**, *380* (9846), 994-1000.
313. Hamilton, N. J.; Kanani, M.; Roebuck, D. J.; Hewitt, R. J.; Cetto, R.; Culme-Seymour, E. J.; Toll, E.; Bates, A. J.; Comerford, A. P.; McLaren, C. A.; Butler, C. R.; Crowley, C.; McIntyre, D.; Sebire, N. J.; Janes, S. M.; O'Callaghan, C.; Mason, C.; De Coppi, P.; Lowdell, M. W.; Elliott, M. J.; Birchall, M. A., Tissue-Engineered Tracheal Replacement in a Child: A 4-Year Follow-Up Study. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **2015**, *15* (10), 2750-7.
314. Elliott, M. J.; Butler, C. R.; Varanou-Jenkins, A.; Partington, L.; Carvalho, C.; Samuel, E.; Crowley, C.; Lange, P.; Hamilton, N. J.; Hynds, R. E.; Ansari, T.; Sibbons, P.; Fierens, A.; McLaren, C.; Roebuck, D.; Wallis, C.; Muthialu, N.; Hewitt, R.; Crabbe, D.; Janes, S. M.; De Coppi, P.; Lowdell, M. W.; Birchall, M. A., Tracheal Replacement Therapy with a Stem Cell-Seeded Graft: Lessons from Compassionate Use Application of a GMP-Compliant Tissue-Engineered Medicine. *Stem cells translational medicine* **2017**, *6* (6), 1458-1464.
315. Zopf, D. A.; Hollister, S. J.; Nelson, M. E.; Ohye, R. G.; Green, G. E., Bioresorbable airway splint created with a three-dimensional printer. *The New England journal of medicine* **2013**, *368* (21), 2043-5.
316. Morrison, R. J.; Sengupta, S.; Flanagan, C. L.; Ohye, R. G.; Hollister, S. J.; Green, G. E., Treatment of Severe Acquired Tracheomalacia With a Patient-Specific, 3D-Printed, Permanent Tracheal Splint. *JAMA otolaryngology-- head & neck surgery* **2017**, *143* (5), 523-525.
317. Huang, L.; Wang, L.; He, J.; Zhao, J.; Zhong, D.; Yang, G.; Guo, T.; Yan, X.; Zhang, L.; Li, D.; Cao, T.; Li, X., Tracheal suspension by using 3-dimensional printed personalized scaffold in a patient with tracheomalacia. *Journal of thoracic disease* **2016**, *8* (11), 3323-3328.
318. Guibert, N.; Didier, A.; Moreno, B.; Mhanna, L.; Brouchet, L.; Plat, G.; Hermant, C.; Mazieres, J., Treatment of post-transplant complex airway stenosis with a three-dimensional, computer-assisted customized airway stent. *American journal of respiratory and critical care medicine* **2017**, *195* (7), e31-e33.
319. Shieh, H. F.; Jennings, R. W., Three-dimensional printing of external airway splints for tracheomalacia. *Journal of thoracic disease* **2017**, *9* (3), 414-416.
320. Ferkol, T.; Schraufnagel, D., The global burden of respiratory disease. *Annals of the American Thoracic Society* **2014**, *11* (3), 404-406.
321. Simkovich, S. M.; Goodman, D.; Roa, C.; Crocker, M. E.; Gianella, G. E.; Kirenga, B. J.; Wise, R. A.; Checkley, W., The health and social implications of household air pollution and respiratory diseases. *NPJ primary care respiratory medicine* **2019**, *29* (1), 1-17.

322. Zar, H. J.; Ferkol, T. W., The global burden of respiratory disease-impact on child health. *Pediatric pulmonology* **2014**, *49* (5), 430-4.
323. Chang, L. H.; Rivera, M. P., Respiratory diseases: meeting the challenges of screening, prevention, and treatment. *North Carolina medical journal* **2013**, *74* (5), 385-92.
324. Borghardt, J. M.; Kloft, C.; Sharma, A., Inhaled therapy in respiratory disease: the complex interplay of pulmonary kinetic processes. *Canadian respiratory journal* **2018**, *2018*.
325. Susewind, J.; de Souza Carvalho-Wodarz, C.; Repnik, U.; Collnot, E. M.; Schneider-Daum, N.; Griffiths, G. W.; Lehr, C. M., A 3D co-culture of three human cell lines to model the inflamed intestinal mucosa for safety testing of nanomaterials. *Nanotoxicology* **2016**, *10* (1), 53-62.
326. Shreffler, J. W.; Pullan, J. E.; Dailey, K. M.; Mallik, S.; Brooks, A. E., Overcoming hurdles in nanoparticle clinical translation: The influence of experimental design and surface modification. *International journal of molecular sciences* **2019**, *20* (23), 6056.
327. Langhans, S. A., Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol* **2018**, *9*, 6-6.
328. Caliri, S. R.; Ramirez, M. A.; Harley, B. A. C., The development of collagen-GAG scaffold-membrane composites for tendon tissue engineering. *Biomaterials* **2011**, *32* (34), 8990-8998.
329. O'Leary, C.; O'Brien, F. J.; Cryan, S.-A., Retinoic Acid-Loaded Collagen-Hyaluronate Scaffolds: A Bioactive Material for Respiratory Tissue Regeneration. *ACS Biomaterials Science & Engineering* **2017**, *3* (7), 1381-1393.
330. Haugh, M. G.; Murphy, C. M.; McKiernan, R. C.; Altenbuchner, C.; O'Brien, F. J., Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds. *Tissue engineering. Part A* **2011**, *17* (9-10), 1201-8.
331. Haugh, M. G.; Jaasma, M. J.; O'Brien, F. J., The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds. *Journal of biomedical materials research. Part A* **2009**, *89* (2), 363-9.
332. Damink, L. H. H. O.; Dijkstra, P. J.; Van Luyn, M. J. A.; Van Wachem, P. B.; Nieuwenhuis, P.; Feijen, J., Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. *Biomaterials* **1996**, *17* (8), 765-773.
333. Rezaee, F.; Georas, S. N., Breaking barriers. New insights into airway epithelial barrier function in health and disease. *American journal of respiratory cell and molecular biology* **2014**, *50* (5), 857-869.
334. Méndez-Echevarría, A.; Fernandez-Prieto, A.; de la Serna, O.; Lopez-Gutierrez, J.-C.; Parron, M.; Marin-Aguilera, B.; Calvo, C., Acute lung toxicity after intralesional bleomycin sclerotherapy. *Pediatrics* **2018**, *141* (1).
335. Pease, J. E.; Sabroe, I., The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. *Am J Respir Med* **2002**, *1* (1), 19-25.
336. Hébert, C. A.; Baker, J. B., Interleukin-8: A Review. *Cancer Investigation* **1993**, *11* (6), 743-750.
337. Caloni, F., Transport of aflatoxin M1 in human intestinal Caco-2/TC7 cells. *Front Pharmacol* **2012**, *3*, 111.
338. Chaudhary, N. I.; Schnapp, A.; Park, J. E., Pharmacologic differentiation of inflammation and fibrosis in the rat bleomycin model. *American journal of respiratory and critical care medicine* **2006**, *173* (7), 769-776.
339. Mouratis, M. A.; Aidinis, V., Modeling pulmonary fibrosis with bleomycin. *Current opinion in pulmonary medicine* **2011**, *17* (5), 355-361.
340. Dirix, L. Y.; Libura, M.; Libura, J.; Vermeulen, P. B.; De Bruijn, E. A.; Van Oosterom, A. T., In vitro toxicity studies with mitomycins and bleomycin on endothelial cells. *Anti-cancer drugs* **1997**, *8* (9), 859-868.
341. Chen, H.; Matsumoto, K.; Brockway, B. L.; Rackley, C. R.; Liang, J.; Lee, J.-H.; Jiang, D.; Noble, P. W.; Randell, S. H.; Kim, C. F.; Stripp, B. R., Airway Epithelial Progenitors Are Region Specific and Show Differential Responses to Bleomycin-Induced Lung Injury. *Stem cells (Dayton, Ohio)* **2012**, *30* (9), 1948-1960.
342. Shi, Y.; Dong, M.; Zhou, Y.; Li, W.; Gao, Y.; Han, L.; Chen, M.; Lin, H.; Zuo, W.; Jin, F., Distal airway stem cells ameliorate bleomycin-induced pulmonary fibrosis in mice. *Stem Cell Research & Therapy* **2019**, *10* (1), 161.
343. Narai, A.; Arai, S.; Shimizu, M., Rapid decrease in transepithelial electrical resistance of human intestinal Caco-2 cell monolayers by cytotoxic membrane perturbants. *Toxicology in vitro* **1997**, *11* (4), 347-354.
344. Duval, K.; Grover, H.; Han, L.-H.; Mou, Y.; Pegoraro, A. F.; Fredberg, J.; Chen, Z., Modeling Physiological Events in 2D vs. 3D Cell Culture. *Physiology* **2017**, *32* (4), 266-277.

345. Osei, E. T.; Booth, S.; Hackett, T.-L., What Have In Vitro Co-Culture Models Taught Us about the Contribution of Epithelial-Mesenchymal Interactions to Airway Inflammation and Remodeling in Asthma? *Cells* **2020**, *9* (7).
346. Papazian, D.; Würtzen, P. A.; Hansen, S. W., Polarized Airway Epithelial Models for Immunological Co-Culture Studies. *International archives of allergy and immunology* **2016**, *170* (1), 1-21.
347. Bisig, C.; Voss, C.; Petri-Fink, A.; Rothen-Rutishauser, B. J. T. i. V., The crux of positive controls-Pro-inflammatory responses in lung cell models. **2019**, *54*, 189-193.
348. Milam, J. E.; Keshamouni, V. G.; Phan, S. H.; Hu, B.; Gangireddy, S. R.; Hogaboam, C. M.; Standiford, T. J.; Thannickal, V. J.; Reddy, R. C., PPAR- γ agonists inhibit profibrotic phenotypes in human lung fibroblasts and bleomycin-induced pulmonary fibrosis. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **2008**, *294* (5), L891-L901.
349. Van Norman, G. A., Limitations of Animal Studies for Predicting Toxicity in Clinical Trials: Is it Time to Rethink Our Current Approach? *JACC Basic Transl Sci* **2019**, *4* (7), 845-854.
350. Cao, X.; Coyle, J. P.; Xiong, R.; Wang, Y.; Heflich, R. H.; Ren, B.; Gwinn, W. M.; Hayden, P.; Rojanasakul, L., Invited review: human air-liquid-interface organotypic airway tissue models derived from primary tracheobronchial epithelial cells—overview and perspectives. *In Vitro Cellular & Developmental Biology - Animal* **2020**.
351. Upadhyay, S.; Palmberg, L., Air-liquid interface: relevant in vitro models for investigating air pollutant-induced pulmonary toxicity. *Toxicological Sciences* **2018**, *164* (1), 21-30.
352. Kim, J. S.; Peters, T. M.; O'Shaughnessy, P. T.; Adamcakova-Dodd, A.; Thorne, P. S., Validation of an in vitro exposure system for toxicity assessment of air-delivered nanomaterials. *Toxicology in vitro* **2013**, *27* (1), 164-173.
353. Cao, Y.; Li, S.; Chen, J., Modeling better in vitro models for the prediction of nanoparticle toxicity: A review. *Toxicology Mechanisms and Methods* **2021**, *31* (1), 1-17.
354. Shamir, E. R.; Ewald, A. J., Three-dimensional organotypic culture: experimental models of mammalian biology and disease. *Nature Reviews Molecular Cell Biology* **2014**, *15* (10), 647-664.
355. Montefusco-Pereira, C. V.; Horstmann, J. C.; Ebsen, T.; Beisswenger, C.; Bals, R.; Guzmán, C. A.; Schneider-Daum, N.; de Souza Carvalho-Wodarz, C.; Lehr, C.-M., P. aeruginosa Infected 3D Co-Culture of Bronchial Epithelial Cells and Macrophages at Air-Liquid Interface for Preclinical Evaluation of Anti-Infectives. *JoVE (Journal of Visualized Experiments)* **2020**, (160), e61069.
356. Kobayashi, K.; Nomoto, Y.; Suzuki, T.; Tada, Y.; Miyake, M.; Hazama, A.; Kanemaru, S.; Nakamura, T.; Omori, K., Effect of fibroblasts on tracheal epithelial regeneration in vitro. *Tissue engineering* **2006**, *12* (9), 2619-2628.
357. Forbes, B.; Ehrhardt, C. J. E. j. o. p.; biopharmaceutics, Human respiratory epithelial cell culture for drug delivery applications. **2005**, *60* (2), 193-205.
358. Lin, H.; Li, H.; Cho, H.-J.; Bian, S.; Roh, H.-J.; Lee, M.-K.; Kim, J. S.; Chung, S.-J.; Shim, C.-K.; Kim, D.-D., Air-liquid interface (ALI) culture of human bronchial epithelial cell monolayers as an in vitro model for airway drug transport studies. *Journal of pharmaceutical sciences* **2007**, *96* (2), 341-350.
359. Li, N.; Wang, D.; Sui, Z.; Qi, X.; Ji, L.; Wang, X.; Yang, L. J. T. E. P. C. M., Development of an improved three-dimensional in vitro intestinal mucosa model for drug absorption evaluation. **2013**, *19* (9), 708-719.
360. Srinivasan, B.; Kolli, A. R.; Esch, M. B.; Abaci, H. E.; Shuler, M. L.; Hickman, J. J., TEER measurement techniques for in vitro barrier model systems. *Journal of laboratory automation* **2015**, *20* (2), 107-126.
361. Macedo, M. H.; Martinez, E.; Barrias, C. C.; Sarmiento, B., Development of an improved 3D in vitro intestinal model to perform permeability studies of paracellular compounds. *Frontiers in bioengineering and biotechnology* **2020**, *8*, 1076.
362. Gumuser, G.; Vural, K.; Varol, T.; Parlak, Y.; Tuglu, I.; Topal, G.; Sayit, E., Assessment of lung toxicity caused by bleomycin and amiodarone by Tc-99m HMPAO lung scintigraphy in rats. *Ann Nucl Med* **2013**, *27* (7), 592-599.
363. Ohta, H.; Chiba, S.; Ebina, M.; Furuse, M.; Nukiwa, T., Altered expression of tight junction molecules in alveolar septa in lung injury and fibrosis. *American journal of physiology. Lung cellular and molecular physiology* **2012**, *302* (2), L193-205.
364. Aoshiba, K.; Tsuji, T.; Nagai, A., Bleomycin induces cellular senescence in alveolar epithelial cells. **2003**, *22* (3), 436-443.

365. Wallach-Dayana, S. B.; Izbiński, G.; Cohen, P. Y.; Gerstl-Golan, R.; Fine, A.; Breuer, R., Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **2006**, 290 (4), L790-L796.
366. Darling, N. J.; Mobbs, C. L.; González Hau, A. L.; Freer, M. A.; Przyborski, S., Bioengineering novel in vitro co-culture models that represent the human intestinal mucosa with improved Caco-2 structure and barrier function. *Frontiers in Bioengineering and Biotechnology* **2020**, 8, 992.
367. Paw, M.; Wnuk, D.; Jakiela, B.; Bochenek, G.; Śladek, K.; Madeja, Z.; Michalik, M., Responsiveness of human bronchial fibroblasts and epithelial cells from asthmatic and non-asthmatic donors to the transforming growth factor- β 1 in epithelial-mesenchymal trophic unit model. *BMC Molecular and Cell Biology* **2021**, 22 (1), 1-14.
368. Adcock, A. F.; Trivedi, G.; Edmondson, R.; Spearman, C.; Yang, L., Three-Dimensional (3D) cell cultures in cell-based assays for in-vitro evaluation of anticancer drugs. *Journal of Analytical & Bioanalytical Techniques* **2015**, 6 (3), 1.
369. Wu, Z.; Guan, R.; Tao, M.; Lyu, F.; Cao, G.; Liu, M.; Gao, J., Assessment of the toxicity and inflammatory effects of different-sized zinc oxide nanoparticles in 2D and 3D cell cultures. *RSC advances* **2017**, 7 (21), 12437-12445.
370. Huang, S.; Wiszniewski, L.; Constant, S., *The use of in vitro 3D cell models in drug development for respiratory diseases*. chapter: 2011; Vol. 8.
371. Skibinski, G.; Elborn, J. S.; Ennis, M., Bronchial epithelial cell growth regulation in fibroblast cocultures: the role of hepatocyte growth factor. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **2007**, 293 (1), L69-L76.
372. Huh, D.; Hamilton, G. A.; Ingber, D. E., From 3D cell culture to organs-on-chips. *Trends in cell biology* **2011**, 21 (12), 745-754.
373. Tanabe, T.; Rubin, B. K., Airway goblet cells secrete pro-inflammatory cytokines, chemokines, and growth factors. *Chest* **2016**, 149 (3), 714-720.
374. Roh, T. T.; Chen, Y.; Paul, H. T.; Guo, C.; Kaplan, D. L., 3D bioengineered tissue model of the large intestine to study inflammatory bowel disease. *Biomaterials* **2019**, 225, 119517.
375. Chow, A. W.-m.; Liang, J. F.-t.; Wong, J. S.-c.; Fu, Y.; Tang, N. L.-s.; Ko, W.-h., Polarized secretion of interleukin (IL)-6 and IL-8 by human airway epithelia 16HBE14o-cells in response to cationic polypeptide challenge. *PloS one* **2010**, 5 (8), e12091.
376. Schröder, S.; Kriesen, S.; Paape, D.; Hildebrandt, G.; Manda, K., Modulation of inflammatory reactions by low-dose ionizing radiation: Cytokine release of murine endothelial cells is dependent on culture conditions. *Journal of immunology research* **2018**, 2018.
377. Sh, G. S.; Petrovskaya, L. E.; Shingarova, L. N.; Svirschevskaya, E. V.; Dolgikh, D. A.; Kirpichnikov, M. P., The effect of TNF and VEGF on the properties of Ea. hy926 endothelial cells in a model of multi-cellular spheroids. *Acta Naturae (англоязычная версия)* **2018**, 10 (1 (36)).
378. Wang, B.; Chen, R.; Gao, H.; Lv, X.; Chen, L.; Wang, W.; Liu, Y.; Zheng, N.; Lin, R., A comparative study unraveling the effects of TNF- α stimulation on endothelial cells between 2D and 3D culture. *Biomedical Materials* **2020**, 15 (6), 065018.
379. Wang, F.; Weaver, V. M.; Petersen, O. W.; Larabell, C. A.; Dedhar, S.; Briand, P.; Lupu, R.; Bissell, M. J., Reciprocal interactions between β 1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proceedings of the National Academy of Sciences* **1998**, 95 (25), 14821-14826.
380. Boyd, D. F.; Thomas, P. G., Towards integrating extracellular matrix and immunological pathways. *Cytokine* **2017**, 98, 79-86.
381. Baker, B. M.; Chen, C. S., Deconstructing the third dimension—how 3D culture microenvironments alter cellular cues. *Journal of cell science* **2012**, 125 (13), 3015-3024.
382. Ball, S. L.; Mann, D. A.; Wilson, J. A.; Fisher, A. J., The role of the fibroblast in inflammatory upper airway conditions. *The American journal of pathology* **2016**, 186 (2), 225-233.
383. Balogh Sivas, K.; Sivas, U.; Hornberg, E.; Zhang, H.; Brändén, L.; Bonfante, R.; Huang, S.; Constant, S.; Robinson, I.; Betts, C. J., A 3D human airway model enables prediction of respiratory toxicity of inhaled drugs in vitro. *Toxicological Sciences* **2018**, 162 (1), 301-308.
384. Rau, J. L., The inhalation of drugs: advantages and problems. *Respiratory care* **2005**, 50 (3), 367-382.
385. Bardoliwala, D.; Javia, A.; Ghosh, S.; Misra, A.; Sawant, K., Formulation and clinical perspectives of inhalation-based nanocarrier delivery: a new archetype in lung cancer treatment. *Therapeutic Delivery* **2021**, 12 (5), 397-418.
386. Kim, N.; Duncan, G. A.; Hanes, J.; Suk, J. S., Barriers to inhaled gene therapy of obstructive lung diseases: A review. *Journal of Controlled Release* **2016**, 240, 465-488.
387. Ecobichon, D. J., *The basis of toxicity testing*. CRC press: 1997.

388. Russell, W. M. S.; Burch, R. L., *The principles of humane experimental technique*. Methuen: 1959.
389. Guillon, A.; Sécher, T.; Dailey, L. A.; Vecellio, L.; De Monte, M.; Si-Tahar, M.; Diot, P.; Page, C. P.; Heuzé-Vourc'h, N., Insights on animal models to investigate inhalation therapy: relevance for biotherapeutics. *International journal of pharmaceutics* **2018**, 536 (1), 116-126.
390. Pankevich, D. E.; Wizemann, T. M.; Mazza, A.-M.; Altevogt, B. M., *International animal research regulations: impact on neuroscience research: workshop summary*. National Academies Press Washington, DC: 2012.
391. National Academies of Sciences, E.; Medicine, *Advancing disease modeling in animal-based research in support of precision medicine: proceedings of a workshop*. National Academies Press: 2018.
392. Schneider-Daum, N.; Carius, P.; Horstmann, J. C.; Lehr, C.-M., Reconstituted 2D cell and tissue models. In *Pharmaceutical Inhalation Aerosol Technology*, CRC Press: 2019; pp 627-651.
393. Huber, J. M.; Amann, A.; Koeck, S.; Lorenz, E.; Kelm, J. M.; Obexer, P.; Zwierzina, H.; Gamerith, G., Evaluation of assays for drug efficacy in a three-dimensional model of the lung. *Journal of cancer research and clinical oncology* **2016**, 142 (9), 1955-1966.
394. Movia, D.; Prina-Mello, A.; Bazou, D.; Volkov, Y.; Giordani, S., Screening the cytotoxicity of single-walled carbon nanotubes using novel 3D tissue-mimetic models. *ACS nano* **2011**, 5 (11), 9278-9290.
395. Klein, S. G.; Serchi, T.; Hoffmann, L.; Blömeke, B.; Gutleb, A. C., An improved 3D tetraculture system mimicking the cellular organisation at the alveolar barrier to study the potential toxic effects of particles on the lung. *Particle and fibre toxicology* **2013**, 10 (1), 1-18.
396. Kasper, J.; Hermanns, M. I.; Bantz, C.; Maskos, M.; Stauber, R.; Pohl, C.; Unger, R. E.; Kirkpatrick, J. C., Inflammatory and cytotoxic responses of an alveolar-capillary coculture model to silica nanoparticles: comparison with conventional monocultures. *Particle and fibre toxicology* **2011**, 8 (1), 1-16.
397. Edwards, J.; Belvisi, M.; Dahlen, S.-E.; Holgate, S.; Holmes, A., Human tissue models for a human disease: what are the barriers? *Thorax* **2015**, 70 (7), 695-697.
398. Konar, D.; Devarasetty, M.; Yildiz, D. V.; Atala, A.; Murphy, S. V., Lung-on-a-chip technologies for disease modeling and drug development: Supplementary issue: Image and video acquisition and processing for clinical applications. *Biomedical engineering and computational biology* **2016**, 7, BECB-S34252.
399. Cui, Y.-n.; Xu, Q.-x.; Davoodi, P.; Wang, D.-p.; Wang, C.-H., Enhanced intracellular delivery and controlled drug release of magnetic PLGA nanoparticles modified with transferrin. *Acta Pharmacologica Sinica* **2017**, 38 (6), 943-953.
400. Palanikumar, L.; Al-Hosani, S.; Kalmouni, M.; Nguyen, V. P.; Ali, L.; Pasricha, R.; Barrera, F. N.; Magzoub, M., pH-responsive high stability polymeric nanoparticles for targeted delivery of anticancer therapeutics. *Communications biology* **2020**, 3 (1), 1-17.
401. Jampilek, J.; Kralova, K., Advances in drug delivery nanosystems using graphene-based materials and carbon nanotubes. *Materials* **2021**, 14 (5), 1059.
402. Walsh, D. P.; Murphy, R. D.; Panarella, A.; Raftery, R. M.; Cavanagh, B.; Simpson, J. C.; O'Brien, F. J.; Heise, A.; Cryan, S.-A., Bioinspired star-shaped poly (L-lysine) polypeptides: efficient polymeric nanocarriers for the delivery of DNA to mesenchymal stem cells. *Molecular pharmaceutics* **2018**, 15 (5), 1878-1891.
403. Walsh, D. P.; Raftery, R. M.; Murphy, R.; Chen, G.; Heise, A.; O'Brien, F. J.; Cryan, S.-A., Gene activated scaffolds incorporating star-shaped polypeptide-pDNA nanomedicines accelerate bone tissue regeneration in vivo. *Biomaterials Science* **2021**.
404. O'Dwyer, J.; Murphy, R.; Dolan, E. B.; Kovarova, L.; Pravda, M.; Velebny, V.; Heise, A.; Duffy, G. P.; Cryan, S. A., Development of a nanomedicine-loaded hydrogel for sustained delivery of an angiogenic growth factor to the ischaemic myocardium. *Drug delivery and translational research* **2020**, 10 (2), 440-454.
405. Mitchell, M. J.; Billingsley, M. M.; Haley, R. M.; Wechsler, M. E.; Peppas, N. A.; Langer, R., Engineering precision nanoparticles for drug delivery. *Nature Reviews Drug Discovery* **2021**, 20 (2), 101-124.
406. Hua, S.; De Matos, M. B. C.; Metselaar, J. M.; Storm, G., Current trends and challenges in the clinical translation of nanoparticulate nanomedicines: pathways for translational development and commercialization. *Front Pharmacol* **2018**, 9, 790.
407. Mura, S.; Hillaireau, H.; Nicolas, J.; Le Droumaguet, B.; Gueutin, C.; Zanna, S.; Tsapis, N.; Fattal, E., Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells. *International journal of nanomedicine* **2011**, 6, 2591.

408. Rotoli, B. M.; Gatti, R.; Movia, D.; Bianchi, M. G.; Di Cristo, L.; Fenoglio, I.; Sonvico, F.; Bergamaschi, E.; Prina-Mello, A.; Bussolati, O., Identifying contact-mediated, localized toxic effects of MWCNT aggregates on epithelial monolayers: a single-cell monitoring toxicity assay. *Nanotoxicology* **2015**, *9* (2), 230-241.
409. Byrne, M.; Victory, D.; Hibbitts, A.; Lanigan, M.; Heise, A.; Cryan, S.-A., Molecular weight and architectural dependence of well-defined star-shaped poly (lysine) as a gene delivery vector. *Biomaterials science* **2013**, *1* (12), 1223-1234.
410. Rao, D. A.; Forrest, M. L.; Alani, A. W. G.; Kwon, G. S.; Robinson, J. R., Biodegradable PLGA based nanoparticles for sustained regional lymphatic drug delivery. *Journal of pharmaceutical sciences* **2010**, *99* (4), 2018-2031.
411. Mura, S.; Hillaireau, H.; Nicolas, J.; Le Droumaguet, B.; Gueutin, C.; Zanna, S.; Tsapis, N.; Fattal, E., Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells. *International journal of nanomedicine* **2011**, *6*, 2591-605.
412. Operti, M. C.; Dölen, Y.; Keulen, J.; van Dinther, E. A. W.; Figdor, C. G.; Tagit, O., Microfluidics-assisted size tuning and biological evaluation of PLGA particles. *Pharmaceutics* **2019**, *11* (11), 590.
413. Gomes dos Reis, L.; Lee, W.-H.; Svolos, M.; Moir, L. M.; Jaber, R.; Windhab, N.; Young, P. M.; Traini, D., Nanotoxicologic effects of plga nanoparticles formulated with a cell-penetrating peptide: Searching for a safe pdna delivery system for the lungs. *Pharmaceutics* **2019**, *11* (1), 12.
414. Peydayesh, M.; Mohammadi, T.; Bakhtiari, O., Effective treatment of dye wastewater via positively charged TETA-MWCNT/PES hybrid nanofiltration membranes. *Separation and Purification Technology* **2018**, *194*, 488-502.
415. Youssry, M.; Al-Ruwaidhi, M.; Zakeri, M.; Zakeri, M., Physical functionalization of multi-walled carbon nanotubes for enhanced dispersibility in aqueous medium. *Emergent Materials* **2020**, 1-8.
416. Gaul, R.; Murphy, R.; McCloskey, A.; De Santi, C.; Ramsey, P. J.; Heise, A.; Greene, C.; Cryan, S.-A., P023 Development of an anti-inflammatory star-polypeptide microRNA delivery system with therapeutic applications in cystic fibrosis. *Journal of Cystic Fibrosis* **2019**, *18*, S63.
417. Mukherjee, M.; Pritchard, D. I.; Bosquillon, C., Evaluation of air-interfaced Calu-3 cell layers for investigation of inhaled drug interactions with organic cation transporters in vitro. *International journal of pharmaceutics* **2012**, *426* (1-2), 7-14.
418. Mills, P. R.; Davies, R. J.; Devalia, J. L., Airway epithelial cells, cytokines, and pollutants. *American journal of respiratory and critical care medicine* **1999**, *160* (supplement_1), S38-S43.
419. Nichols, J. E.; Niles, J. A.; Vega, S. P.; Cortiella, J., Novel in vitro respiratory models to study lung development, physiology, pathology and toxicology. *Stem cell research & therapy* **2013**, *4* (S1), S7.
420. Sanchez-Guzman, D.; Boland, S.; Brookes, O.; Mc Cord, C.; Kuen, R. L.; Sirri, V.; Squiban, A. B.; Devineau, S., Long-term evolution of the epithelial cell secretome in preclinical 3D models of the human bronchial epithelium. *Scientific reports* **2021**, *11* (1), 1-14.
421. Fiegel, J.; Ehrhardt, C.; Schaefer, U. F.; Lehr, C.-M.; Hanes, J., Large porous particle impingement on lung epithelial cell monolayers—toward improved particle characterization in the lung. *Pharmaceutical research* **2003**, *20* (5), 788-796.
422. Tscheik, C.; Blasig, I. E.; Winkler, L., Trends in drug delivery through tissue barriers containing tight junctions. *Tissue barriers* **2013**, *1* (2), e24565.
423. Ruenaroengsak, P.; Chen, S.; Hu, S.; Melbourne, J.; Sweeney, S.; Thorley, A. J.; Skepper, J. N.; Shaffer, M. S. P.; Tetley, T. D.; Porter, A. E., Translocation of functionalized multi-walled carbon nanotubes across human pulmonary alveolar epithelium: dominant role of epithelial type 1 cells. *ACS nano* **2016**, *10* (5), 5070-5085.
424. Di Cristo, L.; Bianchi, M. G.; Chiu, M.; Taurino, G.; Donato, F.; Garzaro, G.; Bussolati, O.; Bergamaschi, E., Comparative in vitro cytotoxicity of realistic doses of benchmark multi-walled carbon nanotubes towards macrophages and airway epithelial cells. *Nanomaterials* **2019**, *9* (7), 982.
425. Banga, A.; Witzmann, F. A.; Petrache, H. I.; Blazer-Yost, B. L., Functional effects of nanoparticle exposure on Calu-3 airway epithelial cells. *Cellular Physiology and Biochemistry* **2012**, *29* (1-2), 197-212.
426. Patient, J. D.; Hajiali, H.; Harris, K.; Abrahamsson, B.; Tannergren, C.; White, L. J.; Ghaemmaghami, A. M.; Williams, P. M.; Roberts, C. J.; Rose, F. R. A. J., Nanofibrous scaffolds support a 3D in vitro permeability model of the human intestinal epithelium. *Front Pharmacol* **2019**, *10*, 456.
427. He, R.-W.; Gerlofs-Nijland, M. E.; Boere, J.; Fokkens, P.; Leseman, D.; Janssen, N. A. H.; Cassee, F. R., Comparative toxicity of ultrafine particles around a major airport in human bronchial epithelial (Calu-3) cell model at the air-liquid interface. *Toxicology in Vitro* **2020**, *68*, 104950.

428. Braakhuis, H. M.; Giannakou, C.; Peijnenburg, W. J. G. M.; Vermeulen, J.; van Loveren, H.; Park, M. V. D. Z., Simple in vitro models can predict pulmonary toxicity of silver nanoparticles. *Nanotoxicology* **2016**, *10* (6), 770-779.
429. Jeong, M. H.; Kim, H. R.; Bang, I. J.; Yoo, S. H.; Lee, S. J.; Lee, K. H.; Chung, K. H., In vitro model for predicting acute inhalation toxicity by using a Calu-3 epithelium cytotoxicity assay. *Journal of pharmacological and toxicological methods* **2019**, *98*, 106576.
430. Antoni, D.; Burckel, H.; Josset, E.; Noel, G., Three-dimensional cell culture: a breakthrough in vivo. *International journal of molecular sciences* **2015**, *16* (3), 5517-5527.
431. Kim, C. O.; Huh, A. J.; Han, S. H.; Kim, J. M., Analysis of cellular senescence induced by lipopolysaccharide in pulmonary alveolar epithelial cells. *Archives of gerontology and geriatrics* **2012**, *54* (2), e35-e41.
432. Xia, T.; Kovochich, M.; Brant, J.; Hotze, M.; Sempf, J.; Oberley, T.; Sioutas, C.; Yeh, J. I.; Wiesner, M. R.; Nel, A. E., Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano letters* **2006**, *6* (8), 1794-1807.
433. Ruenaroengsak, P.; Tetley, T. D., Differential bioreactivity of neutral, cationic and anionic polystyrene nanoparticles with cells from the human alveolar compartment: robust response of alveolar type 1 epithelial cells. *Particle and fibre toxicology* **2015**, *12* (1), 1-20.
434. Nagy, A.; Steinbrück, A.; Gao, J.; Doggett, N.; Hollingsworth, J. A.; Iyer, R., Comprehensive analysis of the effects of CdSe quantum dot size, surface charge, and functionalization on primary human lung cells. *ACS nano* **2012**, *6* (6), 4748-4762.
435. Barosova, H.; Drasler, B.; Petri-Fink, A.; Rothen-Rutishauser, B., Multicellular human alveolar model composed of epithelial cells and primary immune cells for hazard assessment. *JoVE (Journal of Visualized Experiments)* **2020**, (159), e61090.
436. Nagai, H.; Okazaki, Y.; Chew, S. H.; Misawa, N.; Yamashita, Y.; Akatsuka, S.; Ishihara, T.; Yamashita, K.; Yoshikawa, Y.; Yasui, H., Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. *Proceedings of the national academy of sciences* **2011**, *108* (49), E1330-E1338.
437. Yuan, X.; Zhang, X.; Sun, L.; Wei, Y.; Wei, X., Cellular toxicity and immunological effects of carbon-based nanomaterials. *Particle and fibre toxicology* **2019**, *16* (1), 1-27.
438. Fröhlich, E.; Meindl, C., In vitro assessment of chronic nanoparticle effects on respiratory cells. *Nanomaterials—toxicity and risk assessment. Rijeka: InTech* **2015**, 69-91.
439. Higaki, M.; Ishihara, T.; Izumo, N.; Takatsu, M.; Mizushima, Y., Treatment of experimental arthritis with poly (D, L-lactic/glycolic acid) nanoparticles encapsulating betamethasone sodium phosphate. *Annals of the rheumatic diseases* **2005**, *64* (8), 1132-1136.
440. Dailey, L. A.; Jekel, N.; Fink, L.; Gessler, T.; Schmehl, T.; Wittmar, M.; Kissel, T.; Seeger, W., Investigation of the proinflammatory potential of biodegradable nanoparticle drug delivery systems in the lung. *Toxicology and applied pharmacology* **2006**, *215* (1), 100-108.
441. Shipkowski, K. A.; Taylor, A. J.; Thompson, E. A.; Glista-Baker, E. E.; Sayers, B. C.; Messenger, Z. J.; Bauer, R. N.; Jaspers, I.; Bonner, J. C., An allergic lung microenvironment suppresses carbon nanotube-induced inflammasome activation via STAT6-dependent inhibition of caspase-1. *PloS one* **2015**, *10* (6), e0128888.
442. Erdely, A.; Liston, A.; Salmen-Muniz, R.; Hulderman, T.; Young, S.-H.; Zeidler-Erdely, P. C.; Castranova, V.; Simeonova, P. P., Identification of systemic markers from a pulmonary carbon nanotube exposure. *Journal of occupational and environmental medicine* **2011**, *53*, S80-S86.
443. Mitchell, L. A.; Gao, J.; Wal, R. V.; Gigliotti, A.; Burchiel, S. W.; McDonald, J. D., Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. *Toxicological sciences* **2007**, *100* (1), 203-214.
444. Wu, X.; Tao, P.; Zhou, Q.; Li, J.; Yu, Z.; Wang, X.; Li, J.; Li, C.; Yan, M.; Zhu, Z., IL-6 secreted by cancer-associated fibroblasts promotes epithelial-mesenchymal transition and metastasis of gastric cancer via JAK2/STAT3 signaling pathway. *Oncotarget* **2017**, *8* (13), 20741.
445. She, Y. X.; Yu, Q. Y.; Tang, X. X., Role of interleukins in the pathogenesis of pulmonary fibrosis. *Cell Death Discovery* **2021**, *7* (1), 1-10.
446. Sun, M.; Lee, J.; Chen, Y.; Hoshino, K., Studies of nanoparticle delivery with in vitro bio-engineered microtissues. *Bioactive Materials* **2020**, *5* (4), 924-937.
447. Lee, J.; Lilly, G. D.; Doty, R. C.; Podsiadlo, P.; Kotov, N. A., In vitro toxicity testing of nanoparticles in 3D cell culture. *Small* **2009**, *5* (10), 1213-1221.
448. Sambale, F.; Lavrentieva, A.; Stahl, F.; Blume, C.; Stiesch, M.; Kasper, C.; Bahnemann, D.; Scheper, T., Three dimensional spheroid cell culture for nanoparticle safety testing. *Journal of biotechnology* **2015**, *205*, 120-129.

449. Campbell Jr, D. R.; Senger, C. N.; Ryan, A. L.; Magin, C. M., Engineering Tissue-Informed Biomaterials to Advance Pulmonary Regenerative Medicine. *Frontiers in Medicine* **2021**, *8*, 383.
450. Weiss, D. J., Concise review: current status of stem cells and regenerative medicine in lung biology and diseases. *Stem cells (Dayton, Ohio)* **2014**, *32* (1), 16-25.
451. Buxton, D. B., Nanotechnology in the diagnosis and management of heart, lung and blood diseases. *Expert review of molecular diagnostics* **2007**, *7* (2), 149-160.
452. Nichols, J. E.; Niles, J. A.; Cortiella, J., Design and development of tissue engineered lung: Progress and challenges. *Organogenesis* **2009**, *5* (2), 57-61.
453. Gao, M.; Zhang, H.; Dong, W.; Bai, J.; Gao, B.; Xia, D.; Feng, B.; Chen, M.; He, X.; Yin, M.; Xu, Z.; Witman, N.; Fu, W.; Zheng, J., Tissue-engineered trachea from a 3D-printed scaffold enhances whole-segment tracheal repair. *Scientific Reports* **2017**, *7* (1), 5246.
454. Brand-Saberi, B. E. M.; Schäfer, T., Trachea: Anatomy and Physiology. *Thoracic Surgery Clinics* **2014**, *24* (1), 1-5.
455. Li, D.; Yin, Z.; Liu, Y.; Feng, S.; Liu, Y.; Lu, F.; Xu, Y.; Min, P.; Hou, M.; Li, K. J. A. B., Regeneration of trachea graft with cartilage support, vascularization, and epithelization. **2019**, *89*, 206-216.
456. Abouarab, A. A.; Elsayed, H. H.; Elkhayat, H.; Mostafa, A.; Cleveland, D. C.; El Nori, A., Current solutions for long-segment tracheal reconstruction. *Annals of Thoracic and Cardiovascular Surgery* **2017**, ra-16.
457. Ott, L. M.; Weatherly, R. A.; Detamore, M. S., Overview of tracheal tissue engineering: clinical need drives the laboratory approach. *Annals of biomedical engineering* **2011**, *39* (8), 2091-2113.
458. Nichols, J. E.; Niles, J.; Riddle, M.; Vargas, G.; Schilagard, T.; Ma, L.; Edward, K.; La Francesca, S.; Sakamoto, J.; Vega, S., Production and assessment of decellularized pig and human lung scaffolds. *Tissue Engineering Part A* **2013**, *19* (17-18), 2045-2062.
459. Weymann, A.; Patil, N. P.; Sabashnikov, A.; Korkmaz, S.; Li, S.; Soos, P.; Ishtok, R.; Chaimow, N.; Pätzold, I.; Czerny, N., Perfusion-decellularization of porcine lung and trachea for respiratory bioengineering. *Artificial organs* **2015**, *39* (12), 1024-1032.
460. Luo, X.; Liu, Y.; Zhang, Z.; Tao, R.; Liu, Y.; He, A.; Yin, Z.; Li, D.; Zhang, W.; Liu, W. J. B., Long-term functional reconstruction of segmental tracheal defect by pedicled tissue-engineered trachea in rabbits. **2013**, *34* (13), 3336-3344.
461. Austin, M. J.; Rosales, A. M., Tunable biomaterials from synthetic, sequence-controlled polymers. *Biomaterials science* **2019**, *7* (2), 490-505.
462. Baldwin, A. D.; Kiick, K. L., Polysaccharide-modified synthetic polymeric biomaterials. *Peptide Science: Original Research on Biomolecules* **2010**, *94* (1), 128-140.
463. Sharma, S.; Srivastava, D.; Grover, S.; Sharma, V., Biomaterials in tooth tissue engineering: a review. *Journal of clinical and diagnostic research: JCDR* **2014**, *8* (1), 309.
464. Cao, P. F.; Li, B.; Hong, T.; Townsend, J.; Qiang, Z.; Xing, K.; Vogiatzis, K. D.; Wang, Y.; Mays, J. W.; Sokolov, A. P., Superstretchable, self-healing polymeric elastomers with tunable properties. *Advanced Functional Materials* **2018**, *28* (22), 1800741.
465. Durban, M. M.; Lenhardt, J. M.; Wu, A. S.; Small Iv, W.; Bryson, T. M.; Perez-Perez, L.; Nguyen, D. T.; Gammon, S.; Smay, J. E.; Duoss, E. B., Custom 3D printable silicones with tunable stiffness. *Macromolecular rapid communications* **2018**, *39* (4), 1700563.
466. Patricio, T.; Domingos, M.; Gloria, A.; Bártolo, P., Characterisation of PCL and PCL/PLA scaffolds for tissue engineering. *Procedia Cirp* **2013**, *5*, 110-114.
467. Kim, J. Y.; Cho, D.-W., Blended PCL/PLGA scaffold fabrication using multi-head deposition system. *Microelectronic Engineering* **2009**, *86* (4-6), 1447-1450.
468. Komura, M.; Komura, H.; Kanamori, Y.; Tanaka, Y.; Suzuki, K.; Sugiyama, M.; Nakahara, S.; Kawashima, H.; Hatanaka, A.; Hoshi, K., An animal model study for tissue-engineered trachea fabricated from a biodegradable scaffold using chondrocytes to augment repair of tracheal stenosis. *Journal of pediatric surgery* **2008**, *43* (12), 2141-2146.
469. Komura, M.; Komura, H.; Kanamori, Y.; Tanaka, Y.; Ohatani, Y.; Ishimaru, T.; Sugiyama, M.; Hoshi, K.; Iwanaka, T., Study of mechanical properties of engineered cartilage in an in vivo culture for design of a biodegradable scaffold. *The International journal of artificial organs* **2010**, *33* (11), 775-781.
470. Mäkitie, A. A.; Korpela, J.; Elomaa, L.; Reivonen, M.; Kokkari, A.; Malin, M.; Korhonen, H.; Wang, X.; Salo, J.; Sihvo, E., Novel additive manufactured scaffolds for tissue engineered trachea research. *Acta oto-laryngologica* **2013**, *133* (4), 412-417.
471. Yan, B.; Zhang, Z.; Wang, X.; Ni, Y.; Liu, Y.; Liu, T.; Wang, W.; Xing, H.; Sun, Y.; Wang, J., PLGA-PTMC-cultured bone mesenchymal stem cell scaffold enhances cartilage regeneration in tissue-engineered tracheal transplantation. *Artificial organs* **2017**, *41* (5), 461-469.

472. Wu, T.; Zheng, H.; Chen, J.; Wang, Y.; Sun, B.; Morsi, Y.; El-Hamshary, H.; Al-Deyab, S. S.; Chen, C.; Mo, X., Application of a bilayer tubular scaffold based on electrospun poly(l-lactide-co-caprolactone)/collagen fibers and yarns for tracheal tissue engineering. *Journal of materials chemistry. B* **2017**, 5 (1), 139-150.
473. Zang, M.; Zhang, Q.; Chang, E. I.; Mathur, A. B.; Yu, P., Decellularized tracheal matrix scaffold for tracheal tissue engineering: in vivo host response. *Plastic and reconstructive surgery* **2013**, 132 (4), 549e-559e.
474. Hong, P.; Bezuhly, M.; Graham, M. E.; Gratzner, P. F., Efficient decellularization of rabbit trachea to generate a tissue engineering scaffold biomatrix. *International journal of pediatric otorhinolaryngology* **2018**, 112, 67-74.
475. Sears, N. A.; Seshadri, D. R.; Dhavalikar, P. S.; Cosgriff-Hernandez, E., A Review of Three-Dimensional Printing in Tissue Engineering. *Tissue engineering. Part B, Reviews* **2016**, 22 (4), 298-310.
476. Mao, H.; Yang, L.; Zhu, H.; Wu, L.; Ji, P.; Yang, J.; Gu, Z., Recent advances and challenges in materials for 3D bioprinting. *Progress in Natural Science: Materials International* **2020**.
477. Nocera, A. D.; Comín, R.; Salvatierra, N. A.; Cid, M. P., Development of 3D printed fibrillar collagen scaffold for tissue engineering. *Biomedical microdevices* **2018**, 20 (2), 1-13.
478. Gaetani, R.; Feyen, D. A. M.; Verhage, V.; Slaats, R.; Messina, E.; Christman, K. L.; Giacomello, A.; Doevendans, P. A. F. M.; Sluijter, J. P. G., Epicardial application of cardiac progenitor cells in a 3D-printed gelatin/hyaluronic acid patch preserves cardiac function after myocardial infarction. *Biomaterials* **2015**, 61, 339-348.
479. Liu, F.; Chen, Q.; Liu, C.; Ao, Q.; Tian, X.; Fan, J.; Tong, H.; Wang, X., Natural polymers for organ 3D bioprinting. *Polymers* **2018**, 10 (11), 1278.
480. Bose, S.; Robertson, S. F.; Bandyopadhyay, A., Surface modification of biomaterials and biomedical devices using additive manufacturing. *Acta biomaterialia* **2018**, 66, 6-22.
481. Lee, S. J.; Jo, H. H.; Lim, K. S.; Lim, D.; Lee, S.; Lee, J. H.; Kim, W. D.; Jeong, M. H.; Lim, J. Y.; Kwon, I. K., Heparin coating on 3D printed poly (l-lactic acid) biodegradable cardiovascular stent via mild surface modification approach for coronary artery implantation. *Chemical Engineering Journal* **2019**, 378, 122116.
482. Kosik-Kozioł, A.; Graham, E.; Jaroszewicz, J.; Chlanda, A.; Kumar, P. T. S.; Ivanovski, S.; Swieszkowski, W.; Vaquette, C., Surface modification of 3D printed polycaprolactone constructs via a solvent treatment: impact on physical and osteogenic properties. *ACS Biomaterials Science & Engineering* **2018**, 5 (1), 318-328.
483. Ates, Z.; Heise, A., Functional films from unsaturated poly (macrolactones) by thiol-ene cross-linking and functionalisation. *Polymer Chemistry* **2014**, 5 (8), 2936-2941.
484. Guindani, C.; Frey, M. L.; Simon, J.; Koynov, K.; Schultze, J.; Ferreira, S. R. S.; Araújo, P. H. H.; de Oliveira, D.; Wurm, F. R.; Mailänder, V., Covalently binding of bovine serum albumin to unsaturated poly (globalide-Co-ε-Caprolactone) nanoparticles by thiol-ene reactions. *Macromolecular bioscience* **2019**, 19 (10), 1900145.
485. Amaral, H. R.; Wilson, J. A.; do Amaral, R. J. F. C.; Pasçu, I.; de Oliveira, F. C. S.; Kearney, C. J.; Freitas, J. C. C.; Heise, A., Synthesis of bilayer films from regenerated cellulose nanofibers and poly (globalide) for skin tissue engineering applications. *Carbohydrate Polymers* **2021**, 252, 117201.
486. Polloni, A. E.; Chiaradia, V.; do Amaral, R. J. F. C.; Kearney, C.; Gorey, B.; de Oliveira, D.; de Oliveira, J. V.; de Araújo, P. H. H.; Sayer, C.; Heise, A., Polyesters with main and side chain phosphoesters as structural motives for biocompatible electrospun fibres. *Polymer Chemistry* **2020**, 11 (12), 2157-2165.
487. Neufurth, M.; Wang, X.; Wang, S.; Steffen, R.; Ackermann, M.; Haep, N. D.; Schröder, H. C.; Müller, W. E. G., 3D printing of hybrid biomaterials for bone tissue engineering: Calcium-polyphosphate microparticles encapsulated by polycaprolactone. *Acta biomaterialia* **2017**, 64, 377-388.
488. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B., Fiji: an open-source platform for biological-image analysis. *Nature methods* **2012**, 9 (7), 676-682.
489. Zhou, Z.-X.; Chen, Y.-R.; Zhang, J.-Y.; Jiang, D.; Yuan, F.-Z.; Mao, Z.-M.; Yang, F.; Jiang, W.-B.; Wang, X.; Yu, J.-K., Facile strategy on hydrophilic modification of poly (ε-caprolactone) scaffolds for assisting tissue-engineered meniscus constructs in vitro. *Front Pharmacol* **2020**, 11.
490. Savin, C. L.; Peptu, C.; Kroneková, Z.; Sedlačík, M.; Mrlik, M.; Sasinková, V.; Peptu, C. A.; Popa, M.; Mosnáček, J., Polyglobalide-based porous networks containing poly (ethylene glycol) structures prepared by photoinitiated thiol-ene coupling. *Biomacromolecules* **2018**, 19 (8), 3331-3342.
491. O'Brien, F. J.; Harley, B. A.; Yannas, I. V.; Gibson, L., Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds. *Biomaterials* **2004**, 25 (6), 1077-1086.

492. O'Brien, F. J.; Harley, B. A.; Yannas, I. V.; Gibson, L. J., The effect of pore size on cell adhesion in collagen-GAG scaffolds. *Biomaterials* **2005**, 26 (4), 433-441.
493. González-Vázquez, A.; Raftery, R. M.; Günbay, S.; Chen, G.; Murray, D. J.; O'Brien, F. J., Accelerating bone healing in vivo by harnessing the age-altered activation of c-Jun N-terminal kinase 3. *Biomaterials* **2021**, 268, 120540.
494. Barreto, S.; Gonzalez-Vazquez, A.; Cameron, A. R.; Cavanagh, B.; Murray, D. J.; O'Brien, F. J., Identification of the mechanisms by which age alters the mechanosensitivity of mesenchymal stromal cells on substrates of differing stiffness: Implications for osteogenesis and angiogenesis. *Acta biomaterialia* **2017**, 53, 59-69.
495. Rowley, J. V.; Wall, P.; Yu, H.; Tronci, G.; Devine, D. A.; Vernon, J. J.; Thornton, P. D., Antimicrobial Dye-Conjugated Polyglobalide-Based Organogels. *ACS Applied Polymer Materials* **2020**, 2 (7), 2927-2933.
496. Chiaradia, V.; Polloni, A. E.; de Oliveira, D.; de Oliveira, J. V.; Araújo, P. H. H.; Sayer, C., Polyester nanoparticles from macrolactones via miniemulsion enzymatic ring-opening polymerization. *Colloid and Polymer Science* **2018**, 296 (5), 861-869.
497. Gupta, D.; Singh, A. K.; Kar, N.; Dravid, A.; Bellare, J., Modelling and optimization of NaOH-etched 3-D printed PCL for enhanced cellular attachment and growth with minimal loss of mechanical strength. *Materials Science and Engineering: C* **2019**, 98, 602-611.
498. Legrand, C.; Bour, J. M.; Jacob, C.; Capiaumont, J.; Martial, A.; Marc, A.; Wudtke, M.; Kretzmer, G.; Demangel, C.; Duval, D., Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium [corrected]. *Journal of Biotechnology* **1992**, 25 (3), 231-243.
499. Matsiko, A.; Levingstone, T. J.; O'Brien, F. J.; Gleeson, J. P., Addition of hyaluronic acid improves cellular infiltration and promotes early-stage chondrogenesis in a collagen-based scaffold for cartilage tissue engineering. *J Mech Behav Biomed Mater* **2012**, 11, 41-52.
500. Do, A. V.; Khorsand, B.; Geary, S. M.; Salem, A. K., 3D printing of scaffolds for tissue regeneration applications. *Advanced healthcare materials* **2015**, 4 (12), 1742-1762.
501. Sachlos, E.; Czernuszka, J. T., Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. *Eur Cell Mater* **2003**, 5 (29), 39-40.
502. Jammalamadaka, U.; Tappa, K., Recent advances in biomaterials for 3D printing and tissue engineering. *Journal of functional biomaterials* **2018**, 9 (1), 22.
503. Jiao, Z.; Luo, B.; Xiang, S.; Ma, H.; Yu, Y.; Yang, W., 3D printing of HA/PCL composite tissue engineering scaffolds. *Advanced Industrial and Engineering Polymer Research* **2019**, 2 (4), 196-202.
504. Cakmak, A. M.; Unal, S.; Sahin, A.; Oktar, F. N.; Sengor, M.; Ekren, N.; Gunduz, O.; Kalaskar, D. M., 3D Printed Polycaprolactone/Gelatin/BacterialCellulose/Hydroxyapatite Composite Scaffold for Bone Tissue Engineering. *Polymers* **2020**, 12 (9), 1962.
505. Wang, C.; Huang, W.; Zhou, Y.; He, L.; He, Z.; Chen, Z.; He, X.; Tian, S.; Liao, J.; Lu, B., 3D printing of bone tissue engineering scaffolds. *Bioactive materials* **2020**, 5 (1), 82-91.
506. Ates, Z.; Audouin, F.; Harrington, A.; O'Connor, B.; Heise, A., Functional Brush-Decorated Poly (globalide) Films by ARGET-ATRP for Bioconjugation. *Macromolecular bioscience* **2014**, 14 (11), 1600-1608.
507. Rizwan, M.; John, W. T.; Nori, A.; Leong, K. W.; Yim, E. K. F., Cell–substrate interactions. *Principles of Regenerative Medicine* **2019**, 437-468.
508. Saracino, E.; Cirillo, V.; Marrese, M.; Guarino, V.; Benfenati, V.; Zamboni, R.; Ambrosio, L., Structural and functional properties of astrocytes on PCL based electrospun fibres. *Materials Science and Engineering: C* **2021**, 118, 111363.
509. Sodupe Ortega, E.; Sanz-Garcia, A.; Escobedo-Lucea, C., Efficient fabrication of polycaprolactone scaffolds for printing hybrid tissue-engineered constructs. *Materials* **2019**, 12 (4), 613.
510. Gee, A. O.; Baker, B. M.; Silverstein, A. M.; Montero, G.; Esterhai, J. L.; Mauck, R. L., Fabrication and evaluation of biomimetic-synthetic nanofibrous composites for soft tissue regeneration. *Cell and tissue research* **2012**, 347 (3), 803-813.
511. de Melo, L. P.; Salmoria, G. V.; Fancello, E. A.; de Mello Roesler, C. R., Effect of injection molding melt temperatures on plga craniofacial plate properties during in vitro degradation. *International journal of biomaterials* **2017**, 2017.
512. Naseri, E.; Butler, H.; MacNevin, W.; Ahmed, M.; Ahmadi, A., Low-temperature solvent-based 3D printing of PLGA: a parametric printability study. *Drug development and industrial pharmacy* **2020**, 46 (2), 173-178.
513. Guo, T.; Lim, C. G.; Noshin, M.; Ringel, J. P.; Fisher, J. P., 3D printing bioactive PLGA scaffolds using DMSO as a removable solvent. *Bioprinting* **2018**, 10, e00038.

514. Mironov, A. V.; Grigoryev, A. M.; Krotova, L. I.; Skaletsky, N. N.; Popov, V. K.; Sevastianov, V. I., 3D printing of PLGA scaffolds for tissue engineering. *Journal of Biomedical Materials Research Part A* **2017**, *105* (1), 104-109.
515. Yu, C.; Zhu, W.; Sun, B.; Mei, D.; Gou, M.; Chen, S., Modulating physical, chemical, and biological properties in 3D printing for tissue engineering applications. *Applied physics reviews* **2018**, *5* (4), 041107.
516. Tinajero-Díaz, E.; de Ilarduya, A. M.; Cavanagh, B.; Heise, A.; Muñoz-Guerra, S., Poly (amino acid)-grafted polymacrolactones. Synthesis, self-assembling and ionic coupling properties. *Reactive and Functional Polymers* **2019**, *143*, 104316.
517. Nair, N. R.; Sekhar, V. C.; Nampoothiri, K. M.; Pandey, A., Biodegradation of biopolymers. In *Current Developments in Biotechnology and Bioengineering*, Elsevier: 2017; pp 739-755.
518. Haugh, M. G.; Murphy, C. M.; O'Brien, F. J., Novel freeze-drying methods to produce a range of collagen–glycosaminoglycan scaffolds with tailored mean pore sizes. *Tissue Engineering Part C: Methods* **2010**, *16* (5), 887-894.
519. Martin, V.; Ribeiro, I. A.; Alves, M. M.; Gonçalves, L.; Claudio, R. A.; Grenho, L.; Fernandes, M. H.; Gomes, P.; Santos, C. F.; Bettencourt, A. F., Engineering a multifunctional 3D-printed PLA-collagen-minocycline-nanoHydroxyapatite scaffold with combined antimicrobial and osteogenic effects for bone regeneration. *Materials Science and Engineering: C* **2019**, *101*, 15-26.
520. Mozdzen, L. C.; Vucetic, A.; Harley, B. A. C., Modifying the strength and strain concentration profile within collagen scaffolds using customizable arrays of poly-lactic acid fibers. *J Mech Behav Biomed Mater* **2017**, *66*, 28-36.
521. Weisgerber, D. W.; Milner, D. J.; Lopez-Lake, H.; Rubessa, M.; Lotti, S.; Polkoff, K.; Hortensius, R. A.; Flanagan, C. L.; Hollister, S. J.; Wheeler, M. B., A mineralized collagen-polycaprolactone composite promotes healing of a porcine mandibular defect. *Tissue Engineering Part A* **2018**, *24* (11-12), 943-954.
522. Bosworth, L. A.; Hu, W.; Shi, Y.; Cartmell, S. H., Enhancing biocompatibility without compromising material properties: an optimised NaOH treatment for electrospun polycaprolactone fibres. *Journal of Nanomaterials* **2019**, *2019*.
523. Rezvanpanah, E.; Anbaran, S. R. G., The effect of crystallinity on cell growth in semi-crystalline microcellular foams by solid-state process: modeling and numerical simulation. *Materials Research Express* **2017**, *4* (11), 115305.
524. Jiang, W.; Shi, J.; Li, W.; Sun, K., Morphology, wettability, and mechanical properties of polycaprolactone/hydroxyapatite composite scaffolds with interconnected pore structures fabricated by a mini-deposition system. *Polymer Engineering & Science* **2012**, *52* (11), 2396-2402.
525. Leszczak, V.; Baskett, D. A.; Papat, K. C., Smooth muscle cell functionality on collagen immobilized polycaprolactone nanowire surfaces. *Journal of functional biomaterials* **2014**, *5* (2), 58-77.
526. Claudino, M.; van der Meulen, I.; Trey, S.; Jonsson, M.; Heise, A.; Johansson, M., Photoinduced thiol–ene crosslinking of globalide/ε-caprolactone copolymers: Curing performance and resulting thermoset properties. *Journal of Polymer Science Part A: Polymer Chemistry* **2012**, *50* (1), 16-24.
527. Guindani, C.; Dozoretz, P.; Venerai, J. G.; da Silva, D. M.; Araujo, P. H. H.; Ferreira, S. R. S.; de Oliveira, D., Enzymatic ring opening copolymerization of globalide and ε-caprolactone under supercritical conditions. *The Journal of Supercritical Fluids* **2017**, *128*, 404-411.
528. Kandaswamy, C.; Balasubramanian, V., Review of adult tracheomalacia and its relationship with chronic obstructive pulmonary disease. *Current opinion in pulmonary medicine* **2009**, *15* (2), 113-9.
529. Yaremchuk, K., Regular tracheostomy tube changes to prevent formation of granulation tissue. *The Laryngoscope* **2003**, *113* (1), 1-10.
530. Neville, W. E.; Bolanowski, P. J. P.; Soltanzadeh, H., Prosthetic reconstruction of the trachea and carina. *The Journal of thoracic and cardiovascular surgery* **1976**, *72* (4), 525-538.
531. Toomes, H.; Mickisch, G.; Vogt-Moykopf, I., Experiences with prosthetic reconstruction of the trachea and bifurcation. *Thorax* **1985**, *40* (1), 32-37.
532. Ayub, A.; Al-Ayoubi, A. M.; Bhora, F. Y., Stents for airway strictures: selection and results. *Journal of thoracic disease* **2017**, *9* (Suppl 2), S116.
533. Ratnovsky, A.; Regev, N.; Wald, S.; Kramer, M.; Naftali, S., Mechanical properties of different airway stents. *Medical engineering & physics* **2015**, *37* (4), 408-415.
534. Lee, J.-Y.; An, J.; Chua, C. K., Fundamentals and applications of 3D printing for novel materials. *Applied materials today* **2017**, *7*, 120-133.

535. Dhasmana, A.; Singh, A.; Rawal, S., Biomedical grafts for tracheal tissue repairing and regeneration "Tracheal tissue engineering: an overview". *Journal of tissue engineering and regenerative medicine* **2020**, *14* (5), 653-672.
536. Park, J. H.; Park, J. Y.; Nam, I.-C.; Ahn, M.; Lee, J. Y.; Choi, S. H.; Kim, S. W.; Cho, D.-W., A rational tissue engineering strategy based on three-dimensional (3D) printing for extensive circumferential tracheal reconstruction. *Biomaterials* **2018**, *185*, 276-283.
537. 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* **2018**, *26* (5), 745-752.
538. Kandi, R.; Pandey, P. M.; Majood, M.; Mohanty, S., Fabrication and characterization of customized tubular scaffolds for tracheal tissue engineering by using solvent based 3D printing on predefined template. *Rapid Prototyping Journal* **2021**, *27* (2), 421-428.
539. Chiang, T.; Pepper, V.; Best, C.; Onwuka, E.; Breuer, C. K., Clinical Translation of Tissue Engineered Trachea Grafts. *The Annals of otology, rhinology, and laryngology* **2016**, *125* (11), 873-885.
540. Kanzaki, M.; Yamato, M.; Hatakeyama, H.; Kohno, C.; Yang, J.; Umemoto, T.; Kikuchi, A.; Okano, T.; Onuki, T., Tissue engineered epithelial cell sheets for the creation of a bioartificial trachea. *Tissue engineering* **2006**, *12* (5), 1275-1283.
541. Maughan, E. F.; Hynds, R. E.; Proctor, T. J.; Janes, S. M.; Elliott, M.; Birchall, M. A.; Lowdell, M. W.; De Coppi, P., Autologous cell seeding in tracheal tissue engineering. *Current stem cell reports* **2017**, *3* (4), 279-289.
542. Yao, Z.-Y.; Feng, B.-W.; Liu, C.-S.; Liu, Y.-M.; Zhou, H.-Y.; Zhang, X.-H.; Jian, M.-Q.; Mo, J.-L.; Liang, Y.-J.; Chen, L., The Application of a Bone Marrow Mesenchymal Stem Cell Membrane in the Vascularization of a Decellularized Tracheal Scaffold. *Stem Cells International* **2021**, *2021*.
543. Tan, Q.; Steiner, R.; Hoerstrup, S. P.; Weder, W. J. E. j. o. c. t. s., Tissue-engineered trachea: History, problems and the future. **2006**, *30* (5), 782-786.
544. Cyranoski, D., Artificial-windpipe surgeon committed misconduct. *Nature News* **2015**, *521* (7553), 406.
545. Berggren, C.; Karabag, S. F., Scientific misconduct at an elite medical institute: The role of competing institutional logics and fragmented control. *Research Policy* **2019**, *48* (2), 428-443.
546. Coppola, B.; Cappetti, N.; Di Maio, L.; Scarfato, P.; Incarnato, L., 3D printing of PLA/clay nanocomposites: influence of printing temperature on printed samples properties. *Materials* **2018**, *11* (10), 1947.
547. Valino, A. D.; Dizon, J. R. C.; Espera Jr, A. H.; Chen, Q.; Messman, J.; Advincula, R. C., Advances in 3D printing of thermoplastic polymer composites and nanocomposites. *Progress in Polymer Science* **2019**, *98*, 101162.
548. Safshekan, F.; Tafazzoli-Shadpour, M.; Abdouss, M.; Shadmehr, M. B.; Ghorbani, F., Investigation of the mechanical properties of the human tracheal cartilage. *Tanaffos* **2017**, *16* (2), 107.
549. Cristovão, A. F.; Sousa, D.; Silvestre, F.; Ropio, I.; Gaspar, A.; Henriques, C.; Velhinho, A.; Baptista, A. C.; Faustino, M.; Ferreira, I., Customized tracheal design using 3D printing of a polymer hydrogel: influence of UV laser cross-linking on mechanical properties. *3D printing in medicine* **2019**, *5* (1), 1-10.
550. Huang, Z.; Wang, L.; Zhang, C.-X.; Cai, Z.-H.; Liu, W.-H.; Li, W.-M.; Ye, S.-G.; Li, X.-F.; Zhao, J.-B., Biomechanical strength dependence on mammalian airway length. *Journal of thoracic disease* **2021**, *13* (2), 918.
551. Wang, J.; Sun, B.; Tian, L.; He, X.; Gao, Q.; Wu, T.; Ramakrishna, S.; Zheng, J.; Mo, X., Evaluation of the potential of rhTGF- β 3 encapsulated P (LLA-CL)/collagen nanofibers for tracheal cartilage regeneration using mesenchymal stems cells derived from Wharton's jelly of human umbilical cord. *Materials Science and Engineering: C* **2017**, *70*, 637-645.
552. Han, Y.; Lian, M.; Wu, Q.; Qiao, Z.; Sun, B.; Dai, K., Effect of pore size on cell behavior using melt electrowritten scaffolds. *Frontiers in Bioengineering and Biotechnology* **2021**, *9*, 495.
553. Bružauskaitė, I.; Bironaitė, D.; Bagdonas, E.; Bernotienė, E., Scaffolds and cells for tissue regeneration: different scaffold pore sizes—different cell effects. *Cytotechnology* **2016**, *68* (3), 355-369.
554. Soletti, L.; Nieponice, A.; Guan, J.; Stankus, J. J.; Wagner, W. R.; Vorp, D. A., A seeding device for tissue engineered tubular structures. *Biomaterials* **2006**, *27* (28), 4863-4870.
555. Perea, H.; Aigner, J.; Hopfner, U.; Wintermantel, E., Direct magnetic tubular cell seeding: a novel approach for vascular tissue engineering. *Cells Tissues Organs* **2006**, *183* (3), 156-165.

556. Wu, Y. F.; Zhang, J.; Gu, Y. Q.; Li, J. X.; Wang, L. C.; Wang, Z. G., Reendothelialization of tubular scaffolds by sedimentary and rotative forces: a first step toward tissue-engineered venous graft. *Cardiovascular Revascularization Medicine* **2008**, 9 (4), 238-247.
557. Hagandora, C. K.; Gao, J.; Wang, Y.; Almarza, A. J., Poly (glycerol sebacate): a novel scaffold material for temporomandibular joint disc engineering. *Tissue Engineering Part A* **2013**, 19 (5-6), 729-737.
558. Issa, R. I.; Engebretson, B.; Rustom, L.; McFetridge, P. S.; Sikavitsas, V. I., The effect of cell seeding density on the cellular and mechanical properties of a mechanostimulated tissue-engineered tendon. *Tissue Engineering Part A* **2011**, 17 (11-12), 1479-1487.
559. Wiet, M. G.; Dharmadhikari, S.; White, A.; Reynolds, S. D.; Johnson, J.; Breuer, C. K.; Chiang, T., Seeding and implantation of a biosynthetic tissue-engineered tracheal graft in a mouse model. *Journal of visualized experiments: JoVE* **2019**, (146).
560. Janardhanan, S.; Wang, M. O.; Fisher, J. P., Coculture strategies in bone tissue engineering: the impact of culture conditions on pluripotent stem cell populations. *Tissue Engineering Part B: Reviews* **2012**, 18 (4), 312-321.
561. Raredon, M. S. B.; Ghaedi, M.; Calle, E. A.; Niklason, L. E., A rotating bioreactor for scalable culture and differentiation of respiratory epithelium. *Cell medicine* **2015**, 7 (3), 109-121.
562. Kaur, G.; Dufour, J. M., Cell lines: Valuable tools or useless artifacts. Taylor & Francis: 2012.
563. Howard, D.; Buttery, L. D.; Shakesheff, K. M.; Roberts, S. J., Tissue engineering: strategies, stem cells and scaffolds. *Journal of anatomy* **2008**, 213 (1), 66-72.
564. Kim, H. J.; Lee, J. H.; Im, G. I., Chondrogenesis using mesenchymal stem cells and PCL scaffolds. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials* **2010**, 92 (2), 659-666.
565. Lloyd-Griffith, C.; McFadden, T. M.; Duffy, G. P.; Unger, R. E.; Kirkpatrick, C. J.; O'Brien, F. J., The pre-vascularisation of a collagen-chondroitin sulphate scaffold using human amniotic fluid-derived stem cells to enhance and stabilise endothelial cell-mediated vessel formation. *Acta biomaterialia* **2015**, 26, 263-273.
566. Ryan, E. J.; Ryan, A. J.; González-Vázquez, A.; Philippart, A.; Ciraldo, F. E.; Hobbs, C.; Nicolosi, V.; Boccaccini, A. R.; Kearney, C. J.; O'Brien, F. J., Collagen scaffolds functionalised with copper-eluting bioactive glass reduce infection and enhance osteogenesis and angiogenesis both in vitro and in vivo. *Biomaterials* **2019**, 197, 405-416.
567. Burney, P. G. J.; Patel, J.; Newson, R.; Minelli, C.; Naghavi, M., Global and regional trends in COPD mortality, 1990–2010. *European Respiratory Journal* **2015**, 45 (5), 1239-1247.
568. Vos, T.; Lim, S. S.; Abbafati, C.; Abbas, K. M.; Abbasi, M.; Abbasifard, M.; Abbasi-Kangevari, M.; Abbastabar, H.; Abd-Allah, F.; Abdelalim, A., Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *The Lancet* **2020**, 396 (10258), 1204-1222.
569. Crystal, R. G., Research opportunities and advances in lung disease. *Jama* **2001**, 285 (5), 612-618.
570. Kerstjens, H. A. M.; Groen, H. J.; van der Bij, W., Respiratory medicine. *Bmj* **2001**, 323 (7325), 1349-1353.
571. Kiley, J. P., Advancing respiratory research. *Chest* **2011**, 140 (2), 497-501.
572. Schmid, O.; Jud, C.; Umehara, Y.; Mueller, D.; Bucholski, A.; Gruber, F.; Denk, O.; Egle, R.; Petri-Fink, A.; Rothen-Rutishauser, B., Biokinetics of aerosolized liposomal ciclosporin in human lung cells in vitro using an air-liquid cell interface exposure system. *Journal of aerosol medicine and pulmonary drug delivery* **2017**, 30 (6), 411-424.
573. Raja, T. I.; Mozafari, M.; Milan, P. B.; Samadikuchaksaraei, A.; Sefat, F., Nanoengineered biomaterials for tracheal replacement. In *Nanoengineered biomaterials for regenerative medicine*, Elsevier: 2019; pp 285-303.
574. Moztarzadeh, S.; Mottaghy, K.; Sefat, F.; Samadikuchaksaraei, A.; Mozafari, M., Nanoengineered biomaterials for lung regeneration. In *Nanoengineered Biomaterials for Regenerative Medicine*, Elsevier: 2019; pp 305-323.
575. Alfaro-Moreno, E.; Torres, V.; Miranda, J.; Martínez, L.; García-Cuellar, C.; Nawrot, T. S.; Vanaudenaerde, B.; Hoet, P.; Ramírez-López, P.; Rosas, I., Induction of IL-6 and inhibition of IL-8 secretion in the human airway cell line Calu-3 by urban particulate matter collected with a modified method of PM sampling. *Environmental research* **2009**, 109 (5), 528-535.
576. Eutamene, H.; Theodorou, V.; Schmidlin, F.; Tondereau, V.; Garcia-Villar, R.; Salvador-Cartier, C.; Chovet, M.; Bertrand, C.; Bueno, L., LPS-induced lung inflammation is linked to increased epithelial permeability: role of MLCK. *European Respiratory Journal* **2005**, 25 (5), 789-796.

577. Seemann, S.; Zohles, F.; Lupp, A., Comprehensive comparison of three different animal models for systemic inflammation. *Journal of biomedical science* **2017**, *24* (1), 1-17.
578. Kim, S. N.; Lee, J.; Yang, H.-S.; Cho, J.-W.; Kwon, S.; Kim, Y.-B.; Her, J.-D.; Cho, K.-H.; Song, C.-W.; Lee, K., Dose-response effects of bleomycin on inflammation and pulmonary fibrosis in mice. *Toxicological research* **2010**, *26* (3), 217-222.
579. Niemeyer, B. F.; Zhao, P.; Tudor, R. M.; Benam, K. H., Advanced microengineered lung models for translational drug discovery. *SLAS DISCOVERY: Advancing Life Sciences R&D* **2018**, *23* (8), 777-789.
580. Elhattab, K.; Sikder, P.; Walker, J. M.; Bottino, M. C.; Bhaduri, S. B., Fabrication and evaluation of 3-D printed PEEK scaffolds containing Macropores by design. *Materials Letters* **2020**, *263*, 127227.
581. Kim, H.; Yang, G. H.; Choi, C. H.; Cho, Y. S.; Kim, G., Gelatin/PVA scaffolds fabricated using a 3D-printing process employed with a low-temperature plate for hard tissue regeneration: Fabrication and characterizations. *International journal of biological macromolecules* **2018**, *120*, 119-127.
582. Porter, J. R.; Henson, A.; Ryan, S.; Popat, K. C., Biocompatibility and Mesenchymal Stem Cell Response to Poly (ϵ -Caprolactone) Nanowire Surfaces for Orthopedic Tissue Engineering. *Tissue Engineering Part A* **2009**, *15* (9), 2547-2559.
583. Sananta, P.; Rahaditya, I.; Imadudin, M. I.; Putera, M. A.; Andarini, S.; Kalsum, U.; Mustamsir, E.; Dradjat, R. S., Collagen scaffold for mesenchymal stem cell from stromal vascular fraction (biocompatibility and attachment study): Experimental paper. *Annals of Medicine and Surgery* **2020**, *59*, 31-34.
584. Park, H. S.; Park, H. J.; Lee, J.; Kim, P.; Lee, J. S.; Lee, Y. J.; Seo, Y. B.; Ajiteru, O.; Lee, O. J.; Park, C. H. J. T. e.; medicine, r., A 4-Axis technique for three-dimensional printing of an artificial trachea. **2018**, *15* (4), 415-425.
585. Zhang, F.; Aquino, G. V.; Dabi, A.; Bruce, E. D., Assessing the translocation of silver nanoparticles using an in vitro co-culture model of human airway barrier. *Toxicology in Vitro* **2019**, *56*, 1-9.
586. Tilley, A. E.; Walters, M. S.; Shaykhiev, R.; Crystal, R. G., Cilia dysfunction in lung disease. *Annual review of physiology* **2015**, *77*, 379-406.
587. Schneider, C. S.; Xu, Q.; Boylan, N. J.; Chisholm, J.; Tang, B. C.; Schuster, B. S.; Henning, A.; Ensign, L. M.; Lee, E.; Adstamomkonkul, P., Nanoparticles that do not adhere to mucus provide uniform and long-lasting drug delivery to airways following inhalation. *Science advances* **2017**, *3* (4), e1601556.
588. Bahadori, M.; Mohammadi, F., Nanomedicine for respiratory diseases. *Tanaffos* **2012**, *11* (4), 18.
589. Zimmerling, A.; Yazdanpanah, Z.; Cooper, D. M. L.; Johnston, J. D.; Chen, X., 3D printing PCL/nHA bone scaffolds: exploring the influence of material synthesis techniques. *Biomaterials research* **2021**, *25* (1), 1-12.
590. Park, S. A.; Lee, S. J.; Seok, J. M.; Lee, J. H.; Kim, W. D.; Kwon, I. K., Fabrication of 3D printed PCL/PEG polyblend scaffold using rapid prototyping system for bone tissue engineering application. *Journal of Bionic Engineering* **2018**, *15* (3), 435-442.
591. Bagheri, A.; Jin, J., Photopolymerization in 3D printing. *ACS Applied Polymer Materials* **2019**, *1* (4), 593-611.
592. Blume, C.; Reale, R.; Held, M.; Loxham, M.; Millar, T. M.; Collins, J. E.; Swindle, E. J.; Morgan, H.; Davies, D. E., Cellular crosstalk between airway epithelial and endothelial cells regulates barrier functions during exposure to double-stranded RNA. *Immunity, inflammation and disease* **2017**, *5* (1), 45-56.
593. Zhang, X.; Jing, H.; Luo, K.; Shi, B.; Luo, Q.; Zhu, Z.; He, X.; Zheng, J., Exosomes from 3T3-J2 promote expansion of tracheal basal cells to facilitate rapid epithelization of 3D-printed double-layer tissue engineered trachea. *Materials Science and Engineering: C* **2021**, *129*, 112371.
594. Desai, T. J.; Cardoso, W. V., Growth factors in lung development and disease: friends or foe? *Respiratory research* **2001**, *3* (1), 1-4.
595. Merckx, G.; Tay, H.; Lo Monaco, M.; van Zandvoort, M.; De Spiegelaere, W.; Lambrichts, I.; Bronckaers, A., Chorioallantoic membrane assay as model for angiogenesis in tissue engineering: Focus on stem cells. *Tissue Engineering Part B: Reviews* **2020**, *26* (6), 519-539.
596. Van Raemdonck, D.; Neyrinck, A.; Cypel, M.; Keshavjee, S., Ex-vivo lung perfusion. *Transplant International* **2015**, *28* (6), 643-656.