Stiffness memory of 3D-TIPS elastomer nanohybrid scaffolds for biologically responsive bespoke tracheal implants

Thesis submitted for the degree of Doctor of Philosophy (Ph.D.) by

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I hereby certify that the work presented in this thesis is the result of my own investigation carried out at the Centre for Biomaterials in Surgical Reconstruction and Regeneration at University College London during the period of 2015-2019. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Advancements in materials science and 3D printing have inspired the development of bespoke stimuli responsive scaffolds as an attempt to handle challenging issues in tracheal tissue engineering, especially epithelialization and re-vascularization. Poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) elastomers were selected for their appealing mechanical properties and in vitro responses with several cell lines. However, manufacturing PUU-POSS into 3D tracheal structures using conventional printing techniques remains challenging. In this thesis, a reverse 3D printing technique, based on controlled thermally-induced phase separation (TIPS) (3D-TIPS) of a PUU-POSS nanohybrid polymer solution and microphase separation of soft and hard segments of PUU-POSS, was developed to manufacture a wide range of soft elastomer scaffolds with hierarchically porous structure and tuneable stiffness. The dynamic changes of structure, mechanical properties and cellular responses to those scaffolds in vitro and in vivo were systematically characterized. The thermoresponsive stiffness softening of the scaffold was observed at body temperature, which is near the crystal-to-rubber phase transition of the soft segments of PUU-POSS. A potential application of a synthetic trachea based on the 3D-TIPS scaffolds was demonstrated. A successful submucosal tissue analogue of the trachea has been developed based on the multi-layered co-culture of human bronchial epithelial cells (hBEpiCs), human bronchial fibroblast cell (hBFs) or human bone-marrow derived mesenchymal stem cells (hBM-MSCs) supported by collagen hydrogel impregnated the scaffolds as matrix, reminiscent of the native tracheobronchial epithelium architecture. Furthermore, cellular responses of using human dermal fibroblasts (HDFs) and hBM-MSCs on the scaffolds and rat animal model proved the different roles of the hierarchical porous structure, initial stiffness and stiffness softening in modulating cell growth and differentiation, tissue ingrowth and vascularization. Overall, thermoresponsive biomimetic scaffolds by 3D-TIPS hold promise for personalized and biologically responsive soft tissue implants and implantable device with better mechanical matches, angiogenesis and tissue integration.
The reconstruction of large tissue defects poses significant challenges faced by many surgical disciplines. Despite the attention given to the development of novel 3D scaffolds for regenerative medicine applications, current products have limited ability to mimic the dynamic mechanobiological nature found in native tissues. In reality, the clinically available implants/scaffolds are made from well-established high-performance engineering materials with well-defined and stable mechanical properties. They are unable to change in response to physico-chemical stimuli present in the microenvironment, decisive in several cellular processes during development and wound healing.

To address these shortcomings, it has been generated a set of polyurethane thermoresponsive stiffness scaffolds by 3D thermally-induced phase separation (3D-TIPS), in that their elastic properties can adapt to the dynamic environment occurring pre and post-surgery. They can provide a stiffer mechanical support during the early stages of implantation and relax afterwards at body temperature under a fixed structural uniformity to match the intrinsic elasticity of the surrounding soft tissues during healing. The 3D-TIPS is a low-cost technique that allows to generate stimuli responsive scaffolds with a short lead time, making them as potential bespoke soft implants for specific tissue reconstruction with potential improvement of the clinical outcome.

Results in this thesis showed that stiffness softening has significant implications in controlling and tuning cell-material interactions of grafts and implants for tracheal tissue engineering applications. This work provides an early insight both in vitro and in vivo of how stiffness softening can be a driving force in regulating not only proliferation and differentiation of human mesenchymal stem cells (hBM-MSCs) but also tissue ingrowth and vascularization, with a clear clinical analogue of the tracheal tissue. Indeed, scaffolds impregnated with a collagen hydrogel. The thermoresponsive stiffness 3D-TIPS technique has been combined into a single bespoke biomimetic platform of the human tracheobronchial epithelium that has been further tested in vitro. Enhanced mucin expression, well developed ciliation and the formation of tight junctions and adherens junctions for a properly maintained barrier function were promoted on these multi-layered scaffolds with a dynamic viscoelastic stiffness nature. Their hierarchically interconnected porous structure can guide the growth of blood vessels in real 3D scales, paving the way to offer a customizable reproducible technology to generate physiologically relevant 3D biomimetic systems to advance the understanding of airway disease and tracheal repair and reconstruction.
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For this thesis, the text of published or submitted articles was integrally adopted. Editorial changes were made for reasons of uniform presentation. References should be made to the original article(s).

**Peer-reviewed journal publications in the pipeline and in submission from my PhD project:**


10. Wu, L. et al. 3D-printed elastomer for a bronchial epithelial joint cartilage analogue of the upper respiratory airway. *Biomaterials* (*ready for submission*) (*Impact factor 8.8*)

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Macrophage phenotype type II  M2
Mesenchymal stem cell chondrogenic differentiation  MCDM
Mesenchymal stem cell chondrogenic differentiation supplement  MCDS
Molecular weight  MW
Osteopontin  SPP1
Osteocalcin  BGLAP
Osteorix  SP7
Paraformaldehyde  PFA
Phosphate-buffered saline  PBS
Phosphorous  P
Picro Sirus Red  PSR
Polyethylene terephthalate  PET
Polyhedral oligomeric silsesquioxane  POSS
Polyurethane  PU
Poly(urea-urethane)  PUU
Polyvinyl alcohol (PVA)

Quantitative reverse transcriptase polymerase chain reaction (qPCR)

Room temperature coagulation and heating (RTC+H)

Scanning electron microscope (SEM)

Sulfated glycosaminoglycans (sGAG)

Thermally induced phase-separation (TIPS)

Tissue culture plastic (TCP)

Ultimate tensile strength (UTS)

X-Ray (XR)

Yes-associated protein (YAP)

4,4’-methylenebis (phenyl isocyanate) (MDI)

4’,6-diamidino-2-phenylindole (DAPI)
1.1 Background

Organ failure and tissue loss have become increasingly inevitable with aging and onset of disease. A variety of surgical transplants are performed annually worldwide to tackle health-related diseases or disorders. For instance, it has been reported that in the US, 18,597 paediatric patients received kidney transplants, 7,127 adult patients underwent liver transplantation, 2,072 patients underwent lung transplantation and 947 pancreatic transplants were performed in 2015 [1-4]. Also, in the UK, 1,000 laryngectomies are performed every year to combat cancer [5]. Although much progress has been made, whole organ transplantation for patients that have experienced large-scale tissue loss or organ failure still presents limitations. One being the shortage of donated organs; another involving the effects of administering immunosuppressive agents for long periods of time [6].

Respiratory diseases are common medical conditions and represent a leading cause of death in the world [7]. For instance, cystic fibrosis and chronic pulmonary diseases alone have been identified as the fifth cause of death and they are predicted to rise in the coming years [8]. As such, gaining an understanding of the mechanisms of airway injury and airway repair is a main focus in the field of respiratory medicine. Nowadays, there is a growing need to develop synthetic respiratory tissues/organs, physiologically representative of the innate airway, for surgical reconstruction and novel therapeutics. These have propelled research to explore the potential of tissue engineering as an alternative option.

Tissue engineering (TE) is a long-established method to design functional substitutes to take the place of damaged or lost tissue. This approach involves extracting undifferentiated cells from a donor patient and integrating it with an artificially engineered scaffold, with the intention of encouraging the new tissue formation at the site of implantation. The interdisciplinary
approach to tissue regeneration is epitomised by Laurencin’s definition, which states that it involves “the integration of materials science and tissue engineering with stem and developmental cell biology and regenerative medicine towards the regeneration of complex tissues, organs, or organ systems” [9]. With better mechanical and functional properties for specifically engineered substitutes, regenerated tissue generally exhibits better healing characteristics and synthetic/alloplastic grafts as soft tissue substitutes. Consequently, TE has opened up an avenue to overcome the shortcomings of conventional treatment options.

With the continuing advancements in TE, it can be optimistically expected that replaced tissues or organs will closely match their original counterparts, in close compliance with the patient’s regenerative capacity. The extracted cells tend to be small in quantity and then increase to a significant number necessary for tissue regeneration, and subsequently differentiate to the desired cell type when proliferation takes place. Scaffold acts as a cell-carrier to produce an environment that is conducive for cell growth and reproduction. It is paramount that the tissue engineered scaffold exhibits a highly controlled three-dimensional (3D) architecture and geometrical cues to meticulously mimic native tissues and organs. 3D printing, a computer aided design (CAD) technique, is well suited to attaining such requirements by depositing materials in an additive manner [10]. This approach has given rise to diverse 3D biomaterials that have desirable biocompatible (i.e. not harmful to surrounding tissue), non-antigenic (i.e. not relating or having the properties of an antigen), and non-carcinogenic (i.e. not causing cancer) properties [11].

Despite numerous attempts by several groups, the creation of a synthetic airway has to date remained elusive; in fact, extremely high morbidity and mortality rates have been reported [12]. Airway regeneration is still in its infancy and only hypothetical. One of the primary issues confronted in tracheal tissue engineering (TTE) is the epithelialization of tracheal scaffolds [13]. Adequate and early re-epithelialization is critical in ensuring that the trachea performs optimally post-transplantation, both as a physical barrier and maintaining its major role in the respiratory system. A well-formed epithelial layer is also essential to prevent ingrowth of scar tissue that can cause airway obstruction [14]. However, in vivo animal studies using polypropylene collagen tracheal scaffolds have shown that it takes at least 1 month for the presence of epithelial cells to be depicted histologically, and up to 8~12 months for complete epithelialization and cartilage formation [15]. Hence, the reason for researchers seeding tracheal grafts with cells prior to transplantation is to serve as a means of compensating for the timeframe required for sufficient re-epithelialization. Although the current approach results in accelerated regeneration of the epithelium, the existence of functionally ciliary, goblet and basal cells may be lacking. Another issue is to achieve sufficient re-vascularization despite the segmental and anastomotic characteristic of blood vessels [16, 17]. An efficient blood supply is compromised when blood vessels are not properly connected with the tracheal graft, and as synthetic tracheal substitutes do not possess their own blood supply or the capacity for angiogenesis, this can result in the failure of the graft integrating with the host’s tissue.
Therefore, trachea replacement is central to this study with much attention being paid to using tissue engineering (TE) as a treatment method.

1.2 Motivation

Initial stiffness and its progression within microenvironment of implanted scaffolds play an important role in tissue engineering, regulating the successful achievement of epithelialization and re-vascularization. In fact, diverse physiological and pathological processes during trachea replacement are related to changes in cellular and extracellular matrix (ECM) stiffness. Tissue engineered scaffolds, as an effective solution, may be regarded as ECM analogues, and as such, should direct desirable cell behaviour within a given geometrical framework in addition to supplying structural support. In fact, little has been reported on the in vivo tissue responses to changes in scaffold stiffness or viscoelasticity. Clinically available synthetic scaffolds and implants are manufactured from a limited repertoire of biomaterials, which are often stronger and stiffer than surrounding tissues with little adaptability or a narrow range of tunable stiffness in response to biological and physical stimuli. Non-degradable polyurethane (PU) elastomers, particularly poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS), can demonstrate a wide range of tunable physical and mechanical properties without changes of their underlying molecular structure, making it a promising material in tracheal tissue engineering (TTE).

An appropriate porous structure can be an effective approach to maintain a scaffold’s material composition whilst reduce stiffness mismatch and is essential to allowing vascularization and tissue ingrowth within the scaffold. The advent of 3D printing has presented the biomedical community with an opportunity to design and manufacture complex 3D organ-like shapes with well-defined macroscopic porous architectures based on patient-specific dimensions. Printing stimuli-responsive materials will create dynamic 3D structures that can transform their shapes or behaviour under various stimuli. Whereas, solution-based PUU-POSS cannot be printed directly due to the high boiling temperatures of dimethylacetamide (DMAC) used as solvent and the slow coagulation process in water, thus it does not easily lend itself to the creation of large porous constructs with complex shapes. Fused deposition modelling (FDM) is one of the most widely used techniques for manufacturing polymer-based scaffolds, and it can be further modified to incorporate different materials processing into a single manufacturing step to overcome some of the issues of conventional printing.

1.3 Layout of this thesis

The focus of this thesis is to develop a bespoke patient specific autologous tissue reconstruction approach via the 3D printing technology. Employing a novel indirect 3D printing guided thermally induced phase-separation (3D-TIPS) technique enables preparation for an elastomeric nanohybrid scaffold. The property of the scaffold allows for initial mechanical support to be given to the surrounding tissues, before gradually softening to a better mechanical
match for the intended environment. This responsive behaviour raises hopes for advancing specialised soft tissue implants that are responsive to biological cues and can promote cell growth and scaffold tissue integration when in application.

For this research, it is significant to ensure that well-defined micro-channels can be digitally programmed to guide the 3D-TIPS of solution-based PUU-POSS and lead to the creation of full-scale scaffolds that have established structural hierarchy, interconnected pores, controlled porosity, crystallinity and mechanical properties. Stiffness softening of the internal structure is characterised by the transformation into a soft rubbery phase at body temperature. This transformation is expected not to impinge on the performance of the scaffold, as the overall structure can serve a better mechanical match for the recipient environment.

The stiffness softening of the scaffold could then be used to demonstrate its influence on the growth and differentiation of human bone-marrow mesenchymal stem cells (hBM-MSCs) into chondrogenic and osteogenic lineages and human bronchial epithelial cells (hBEpiCs). The success of this study would be geared towards developing a physiological representative 3D tracheobronchial model. Although in vitro studies would provide limited insight into the behaviour of the 3D model in the body, its biological interaction with cells during dynamic mechanical relaxation of these scaffolds would demonstrate their potential for in vivo studies.

Apart from Chapter 1 (Introduction), Chapter 2 (General Materials and Methods) and Chapter 7 (Conclusions and Future Perspectives), this thesis consists of four main chapters, which are outlined below with their respective objectives:

a) **Chapter 3: Indirectly 3D Printing Guided Stiffness Softening Elastomeric Nanohybrid Scaffolds for Novel Biologically Responsive Soft Implants**
   - To develop thermoresponsive non-degradable PUU-POSS nanohybrid scaffolds utilising 3D printing guided thermally induced phase-separation (3D-TIPS) technique;
   - To establish the correlations between the structure and properties of the scaffolds in different 3D-TIPS processing conditions;
   - To characterize and uncover the mechanism of the scaffolds with stiffness softening;
   - To determine the biocompatibility of the scaffold by in vitro human dermal fibroblasts (HDFs) interactions with the stiffness softening scaffolds, including fibroblast attachment and proliferation.

b) **Chapter 4: Stiffness Softening of 3D-TIPS Elastomer Nanohybrid Scaffolds Regulating Chondrogenesis and Osteogenesis of Human Bone-Marrow Mesenchymal Stem Cells**
   - To prove the ability to regulate proliferation and differentiation of hBM-MSCs into chondrogenic and osteogenic lineages over a 4-week stiffness relaxation period, by utilising characterisation techniques such as immunohistochemistry, histological, enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR);
• To demonstrate the various preferences of initial stiffness for hBM-MSCs differentiation with enhanced chondrogenesis on the softer scaffold and osteogenesis on the stiffer scaffold.

c) **Chapter 5: Engineering A Tracheobronchial Epithelial Model on 3D-TIPS Thermoresponsive Scaffolds**
- To engineer a bilayered 3D printed soft scaffolds base on collagen and non-degradable PUU-POSS nanohybrid scaffolds for the advancement of a 3D in vitro epithelial co-culture model;
- To show that hBEpiCs are able to attach and grow on the scaffolds adopting an epithelial monolayer;
- To co-culture hBEpiCs with either human bronchial fibroblasts or hBM-MSCs to create a tissue analogue of the upper respiratory tract and thereby validate the 3D printed scaffolds as a platform to support co-culture and cellular organisation;
- To show that these scaffolds can facilitate hBEpiCs differentiation, with enhance mucin expression, develop ciliation and the formation of intercellular tight junctions when compare to untreated PUU-POSS scaffolds or cell culture control via immunohistochemistry, histology, ELISA and qPCR.

d) **Chapter 6: Cellular Responses to Thermoresponsive Stiffness Softening Elastomer Nanohybrid Scaffolds: An In vitro and In vivo Study**
- To investigate the effect of stiffness softening and the hierarchically interconnected porous structure of the scaffolds on mouse embryonic dermal fibroblasts (3T3-J2 cells) in vitro and rat tissue integration in vivo;
- To study angiogenesis of the scaffold in rats;
- To study inflammatory response to the scaffolds during subcutaneous implantation in rats for a 3-month period.

1.4 Literature review

1.4.1 Anatomical structure of the trachea

The trachea is responsible for respiration whilst also providing airway protection [18, 19]. Inadequate care of the trachea can endanger one’s health and result in various diseases or disorders, such as tracheobronchial tuberculosis [20, 21], tracheobronchitis (caused by Aspergillus) [22, 23] and tracheobronchopathia osteochondroplastica [24]. Understanding the anatomical structure of the trachea (see Figure 1.1) is essential to gaining insight of the detrimental effects the disease can present, as well as the possibility of designing a suitable trachea replacement.
Figure 1.1 Image of the structure of the human trachea: (A) anterior view, (B) longitudinal section depicting the action of the mucociliary escalator, and (C) a cross section of the trachea illustrating the C-shaped tracheal cartilage. Reprinted from Netter, F.H. (2010) [25]

The trachea is made up of both fibrous and cartilaginous tissue in varying amounts and takes the position between the larynx and the main bronchus (see Figure 1.1A). It consists of 15–20 C-shaped cartilage rings that are connected by the trachealis muscle [26] (see Figure 1.1C). The trachea serves three functions simultaneously. The first function is to serve as an anatomical role in order to maintain airway patency. The second is for physiological reasons (i.e., humidify inhalation air and clear debris), whilst the third is as a metabolic role (i.e., for fluid and ionic transport) [14]. The trachea is described as a structurally dynamic organ due to its ability to constantly change shape according to the demands of the respiratory cycle [26]. Upon rest, its length is typically 10 cm, however at full inhalation its length can increase up to 15 cm. This degree of efficiency is not just restricted to its length, as its transverse diameter can increase up to 30 % from 2 cm during coughing [26].

The inner trachea is lined with ciliated pseudostratified columnar epithelium (see Figure 1.1B). The epithelium serves as a protective barrier and first line of defence against inhaled hazardous substances in environment, as well as possessing the ability to maintain the homeostasis of the normal epithelium after an injury or during tissue renewal [13]. This layer is made up of ciliary cells, goblet cells and basal cells, that are essential to tracheal function and bear self-healing capabilities [27]. The trachea comprises four layers (Figure 1.1B), where the mucosa
(epithelia and lamina propria) occupies the innermost layer and then follows the submucosa and fibrocartilaginous middle layer, and the fourth layers of adventitia. The mucosa is lined with ciliated pseudostratified columnar epithelia [19, 28]. The cilia are vital for removing debris that is trapped in mucus by upwards motion towards the oropharynx [29]. On the other hand, the submucosa is lined with both loose connective tissue and serous and mucous glands. The latter serves the role of generating mucus that can trap foreign particles. The fibrocartilage layer is located between the submucosa and the adventitia. The cartilage present in the former layer is enclosed with a connective tissue called perichondrium [30]. Finally, the adventitia possesses blood vessels, lymphatic blood vessels and nerves that are key for binding the trachea to the surrounding structures [30].

1.4.2 Clinical need for trachea replacement

Multiple etiologies such as trauma, neoplastic diseases, congenital anomalies, and intubation procedures can result in damaged parts of the trachea that are irrevocable [31]. In order to treat tracheal defects effectively, the defect size and the extent of the damage should be considered. In fact, trachea damage typically exists as small sized defects that can be repaired by segmental resection [32]. An end-to-end anastomosis is a well-known procedure that is opted for when there is severe damage or total trachea stenosis, which results in a defected length of 5 to 6 cm or 50% of the total length of an adult trachea [12, 33, 34]. In the event that a portion of a damaged trachea exceeds the general limits for safe resection, primary anastomosis can be undertaken. In the instance that a resection is inadvisable for a patient, biomaterials such as stents and T tubes can be used in a palliative manner to alleviate the condition. However, these biomaterials are susceptible to erosion and can cause infection or fistula formation that would result in frequent hospital admissions in the foreseeable future [35, 36].

This has created an alternative procedure, such as organ transplantation that may be more feasible. For such a procedure, the viable organ is taken from a donor patient and subsequently surgically implemented in the body of the recipient in need. Trachea transplantation is encouraged in severe cases whereby the complexity of the damage is beyond primary repair. However, its expansion is constrained by the chronic shortage of suitable donors, the need for life-long immunosuppression, and by socioeconomic and religious concerns [6]. The complex anatomical features and the variety of tissues that make up the trachea (i.e., respiratory epithelium, submucosa, cartilage, and blood vessels) also complicate successful transplantation. Difficulty arises particularly when restoring blood supply to the grafted biomaterial [34-37]. This highlights a need for more research surrounding successful tracheal replacements.

1.4.3 Tissue engineering in tracheal replacement

The successful management of long-segment tracheal defects that exceed the limits for primary reconstruction has increased interest in tracheal tissue engineering (TTE). TTE is a promising
approach that is capable of mitigating the challenges mentioned above. Some of the clinical advantages of this approach are that the recipient does not need to be administered immunosuppressant, and that the transplant is capable of promoting novel tissue formation through encouraging cell adhesion and proliferation [38]. TTE approach is made up of three integral parts: cells for seeding, cell growth factors, and the scaffold on to which the cells are seeded.

1.4.3.1 Cells

Namely embryonic cells and adult stem cells are involved in TTE, selecting the appropriate stem cell plays a critical role in TTE processing prior to its seeding with a scaffold and subsequent implantation [39]. The suitable cell type should exhibit stability and functionality following differentiation, and non-immunogenic properties for the purpose of harvesting [40, 41].

Currently, there is no unanimous thought regarding the ideal cell type for TTE. Animal studies have shown that seeding with bone marrow derived cells or progenitor cells may contribute to airway tissue regeneration and may also prevent bacterial contamination [42-44]. Additionally, mesenchymal stem cells (MSCs) are multipotent cells that are easily obtained from recipient tissues without any ethical concerns. Although MSCs are not as easily extracted compared to induced pluripotent stem cells (iPSCs), their accessibility, non-immunogenic properties, a safe track record in clinical practice, and a high retrievable yield make them attractive to use for tracheal replacement [45]. Interestingly, bone-marrow mesenchymal stem cells (BM-MSCs) are comparable in vitro to human vocal fold fibroblasts when assessed by cell viability and cell surface markers [46]. Human vocal fold fibroblasts are not available commercially and the primary cells are difficult to acquire; therefore, BM-MSCs may well find a second use in laryngeal tissue engineering [41]. Currently, autologous BM-MSCs are the most commonly used cell type for tracheal engineering [41, 45]. Madhuri et al. suggested that there are two cellular considerations in TTE: the first being the incorporation of epithelial cells that can line the inside of a trachea, and secondly the introduction of chondrocytes that are integral to the development of hyaline cartilage [47]. As such, epithelial cells and chondrocytes are key for TTE purposes. The former cell type is necessary for lining the airway, whereas the latter is responsible for forming new tracheal cartilage [41, 48, 49].

As mentioned before, ciliated pseudostratified columnar epithelium, which also contains goblet and basal cells, are located in the trachea. The goblet cells form mucosal layers that trap foreign particles such as pathogens and for removal. These cells are also essential in maintaining the water/ion balance and sustaining healthy epithelium following graft implantation [48]. Human nasal epithelial cells show superiority in availability and fertility. However, there is inadequate research detailing that these cells can recreate the pseudo-stratified columnar epithelium [50].

Chondrocytes serve an important role of producing and conserving the ECM of cartilage. Chondrocytes can be found in abundance in cartilage located at the nose, trachea and rib cage
Hyaline cartilage is the main component that forms the U-shaped rings of the trachea. Unlike epithelial cells, extracting and growing chondrocytes is a tedious process [48]. To alleviate this challenge, chondrocytes can be obtained from differentiated BM-MSCs and also epithelial cells. It is noteworthy to mention that chondrocytes cultured in vitro are known to de-differentiate over a period of time, so it is important to seed them with gels like agarose or collagen to counteract this. This is because the 3D environment provided by an agarose or collagen-based gel is a better suited supportive substrate for chondrocyte differentiation into hyaline cartilage [51-53].

1.4.3.2 Growth factors

Growth factors, including erythropoietin, vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), fibroblast growth factor (FGF) and granulocyte colony-stimulating factor (G-CSF), bone morphogenetic proteins-2 (BMP-2) [54-56] are molecules that regulate cell proliferation, differentiation and cell function. Among them, TGF-β has proven to be one of the most promising factors for in vitro engineering of cartilage, with subtypes of TGF-β, i.e. TGF-β3, being an effective selection in promoting BM-MSC chondrogenesis [57]. Growth factors are essential elements in the TTE process and their selection should be considered for successful tissue growth.

1.4.3.3 Scaffold material

For decades, many researchers have dedicated themselves to the selection of a quintessential tracheal scaffold, which is evidenced by several published review papers [48, 58-60]. The suitable scaffold must have several prerequisite properties, such as bioactivity, the ability to host seeded cells, non-immunogenicity (i.e., obstruct a humoral and/or cell-mediated immune responses), non-toxicity and non-carcinogenicity/non-teratogenicity (i.e., not causing cancer/deformity). The scaffold should also imitate the desired tissue environment and possess the required mechanical properties that can be maintained during its application. Mechanical properties of native trachea and implanted scaffolds are detailed in section 1.4.4. Tests that evaluate the air- and liquid-tight seals of the tracheal scaffold are also important to consider so that it can perform optimally. Furthermore, the structural support of the tracheal scaffold should be suited to maintain an open airway [61]. Porosity of the scaffold is another property to consider as it provides an avenue for vascularisation and cellular ingrowth within the host connective tissue [62, 63]. Tissue engineered scaffolds can be constructed from naturally occurring or human-made synthetic materials.

◆ Natural derived scaffold

Decellularization describes a process whereby cells and immunogenic materials are discarded from the ECM. This is done as a means of only retaining the biomechanical integrity of the scaffold. This is significant as certain changes, such as stiffness, could be detrimental to the
differentiation of mesenchymal cells [64]. Collagen is a fundamental component of tracheal ECM, as well as glycosaminoglycans, and elastin. The remaining ECM of the decellularized organ provides the microarchitecture for adhesion, proliferation, remodelling, differentiation and angiogenesis [65, 66]. Decellularized trachea has drawn much attention particularly due to their potential in pre-clinical animal studies and the human clinical setting, as can be seen in Table 1.5. However, mechanical properties should be paid more attention, because several studies have reported a loss of mechanical integrity in decellularized grafts [67, 68]. In addition, there is difficulty in producing decellularized tissue scaffolds in bulk due to limited donor tissues or organs, long processing time, high-cost and contamination risk carriage [49]. Furthermore, despite the process of decellularization alleviating the probability of an immunological response, other unfavourable reactions can be induced due to the formation of damage-associated molecular pattern proteins (DAMPs) [69] or the retention of nucleic acid remnants [70]. These drawbacks suggest that more efforts ought to be invested for seeking alternative solutions.

A better control over surface chemistry and morphology of scaffolds to promote cellular functions, along with tuneable structural and physical properties, can be obtained from polymers [71]. Commonly used natural protein-type polymers include collagen and gelatin, while common natural polysaccharide-type polymers include chitin, chitosan and alginate [72]. In particular, collagen is a fibrin composed of a triple-stranded helical structure maintained by hydrogen and covalent bonds. Collagen type I, collagen type II, and collagen type III, found in bone, cartilage and blood vessel walls, respectively, constitute more than 80% of all collagen in human body [73]. The protein is non-cytotoxic and biocompatible, has cross-linking ability to adjust mechanical, degradation and water absorption properties, but shows inadequacy in elasticity, mechanical strength and dimensional stability [74]. As the most abundant protein found in human beings, collagen can be used to form the basis of a naturally-derived hydrogel, making it a viable biocompatible material for tissue engineering applications [75]. Collagen has been found to be suitable for generating scaffolds for culturing MSC in tissue engineering [76].

Though natural polymers have excellent biocompatibility, able to communicate with cells to promote interactions, and also valued for being nontoxic and low inflammatory reactions [77], they still bear a number of disadvantages, including inadequate material source, complex fabrication process, poor mechanical properties, and uncontrollable biodegradation [78, 79]. On the other hand, preparation of a variety of natural polymers may endanger human from other species of the possibility of disease transmission [80].

**Synthetic scaffold**

The challenges mentioned above can be remitted by employment of synthetic scaffolds. This approach is praised for the versatility in design customisation for the patient's needs, and that following sterilisation is not compromised [49, 59]. In addition, better manipulation of material...
properties such as strength, degradation time, porosity and microstructure can be realized [81]. Particularly, synthetic polymers have found widespread applications in TTE, due to their advantages of more straightforward to control mechanical and chemical properties, readily available, inexpensive to produce and can be non-toxic and compatible with cells [82]. Synthetic polymeric materials can be categorized into degradable and non-degradable materials according to their chemical stability.

➢ Degradable

Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL) and their copolymers such as poly(lactic glycolic) acid (PLGA), are among the most widely used synthetic degradable polymers, with FDA approval for certain human use [83]. PGA is a highly crystalline thermoplastic biodegradable polymer, it has been incorporated into experimental tracheal scaffold reinforcing sponge sheet, where epithelialization and newly formed cartilage in an artificial trachea were investigated [84]. PGA degrades rapidly in aqueous solutions or in vivo and loses its mechanical integrity within 2 and 4 weeks [85], which may not be sufficient time for adequate regeneration of trachea. Comparatively, PLA scaffolds may lose their mechanical integrity after months to years either in vitro or in vivo [85], making it suitable for both in vitro and in vivo applications where long-term mechanical integrity is critical, including TTE [86, 87]. PLGA, on the other hand, has numerous property advantages over its polymeric counterparts. PLGA exhibits mechanical properties equivalent to soft tissues and it is already used as a scaffold for TTE [88]. Compared with other polymers, PCL needs much longer time to degrade completely, ranging from 2 to 4 years [89]. Mechanical and thermal properties, along with degradation profile of PCL make it more attractive for long-term implants, particularly airway tissue engineering (shown in Table 1.4). Despite its good biocompatibility and processability, hydrophobicity requires PCL to conduct surface modification to allow cell attachment and subsequent differentiation [90].

While the residual organic solvents during degradation of degradable materials present potential hazardous risk to cells or human body [91]. As such, poor biostability is not conducive to tissue engineering because implants that require longer retention times or higher surrounding tissue environmental stability to maintain inherent structural integrity [92].

➢ Non-degradable

Non-degradable synthetic polymers have been used in various medical implants for decades. These materials provide long-lasting support in a dynamic environment over time and the ultimate performance of the patient throughout their lifetime, as well as avoiding the problem of asynchronous degradation through new tissue regeneration and degradation of the harmful end products of the polymer [93]. Numerous non-degradable synthetic polymers have been used in biomedical applications such as silicone, polyolefin, polyethylene terephthalate (PET), polytetrafluoroethylene (PTFE), polyurethanes (PUs). Among them, silicone and polyolefin rubber exhibit insufficient durability under long-term stress [94]. PET and PTFE have been used
in the development of vascular grafts, but the lack of bioactivity of PET limits its potential use in tissue engineering [95], while PTFE suffers from extensive wear and biocompatibility issues [96].

Non-degradable PUs have always been a popular choice because they are free to choose different monomers, which makes them available in adjustable mechanical, physical and biological properties, as well as blood and tissue compatibility [97]. PUs are produced by the polycondensation of a poly-isocyanate, a polyol, and usually a chain extender species, characterized by segmented block-copolymers with repeating units containing intramolecular urethane moieties [98]. The polymer inherits both hydrophilic hard segment and hydrophobic soft segment. Microphase separation of the hard and soft segments provides their prominent elastic and mechanical properties, and absorbance of physical stress.

Innovations in non-degradable PUs have been made for long-term implantations, such as poly(urea-urethane) (PUU) [99], polyester urethane urea (PEUU) [100], silk fibroin polyurethane (SFPU) [101]. In particular, PUU has demonstrated reliable and predictable self-assembly through phase separation between its alternating soft and hard segment chains. PUU is an elastomer with a unique nanostructure that offers excellent hyperelasticity and makes it suitable for soft implants and scaffolds. Furthermore, it is relatively biocompatible, nontoxic, and elicits a manageable low inflammatory response in vivo [102]. PUU scaffolds have been fabricated by Lu et al., they found a greater amount of proteoglycans and collagen II were synthesized by the inner annulus fibrous cells cultured in the scaffolds [103]. Şenel Ayaz et al. also cultured the H9C2 cardiac myoblast cell line in the PUU scaffolds, believing that the scaffolds might have potential use in artificial hearts in the future [104].

Poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) is formed based on PUU with the introduction of polyhedral oligomeric silsesquioxane (POSS), which acts as an adjuvant for the polymer [105]. The POSS nanocage with approximate size of 1.5 nm consists of a symmetrical inner inorganic framework of silicon and oxygen atoms, externally covered by organic functional groups, making POSS molecules compatible with PUs [106, 107]. In addition, smaller particle size increases the possibilities to be incorporated into polymeric materials and as such enforce polymers’ mechanical strength, modulus, rigidity [107]. It is featured with excellent hyperelasticity, which makes it a suitable choice for soft implants and scaffolds [94]. The molecular structure of PUU-POSS was illustrated in Figure 1.2.
1.4.4 Mechanical properties of trachea and tracheal scaffolds

Scaffold materials involved in TTE shall possess proper mechanical properties, since failure of some trachea implantations can be attributed to scaffold collapse and fibrosis induced by mismatching of mechanical properties [108]. In addition, material stiffness is responsible for regulating cellular commitment to a specific lineage [109]. As such, a profound knowledge of mechanical properties of native trachea and scaffold material is much needed.

1.4.4.1 Mechanical properties of native trachea

Tracheal cartilage, smooth muscle and annular ligament are the causes of longitudinal extension and lateral stiffness of the native trachea [108, 110]. Although the chest pressure changes during breathing, the ring keeps the trachea unblocked, while the muscles and connective ligaments prevent tracheal collapse by adjusting the diameter and length [111]. Considering porcine trachea model was generally believed to resemble that of human, and the rabbit model is widely used for tracheal reconstruction for its similarity to that of the human infant [112, 113], mechanical properties with respect to human, porcine and rabbit tracheal cartilage were presented in Table 1.1. A large span of equilibrium modulus can be derived, which can be attributed to variations in experimental strategies and individual differences. Mechanical behaviors of native trachea at the tissue and organ levels were detailed in Boazak

Figure 1.2 Schematic diagram of the molecular of poly (urea-urethane) terminated by polyhedral oligomeric silsesquioxane (PUU-POSS), where R=i-Bu is an indicative of isobutyl tertiary group, modelled with Materials Studios (Chemdraw17, Cambridge, UK).
et al. [108].

Table 1.1 Mechanical properties of human and porcine trachea cartilage (adapted from Boazak et al. [108]).

<table>
<thead>
<tr>
<th>Source</th>
<th>Test type</th>
<th>Reported constants</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human, n = 10, age 17–81 years</td>
<td>Uniaxial tensile</td>
<td>Tensile modulus: 1–15 MPa</td>
<td>Rains et al. (1992) [114]</td>
</tr>
<tr>
<td>Human, n = 10, age 17–81 years</td>
<td>Uniaxial tensile</td>
<td>Tensile modulus: 13.6 ± 1.5 MPa (ablumenal superficial zone); 4.6 ± 1.7 MPa (middle zone)</td>
<td>Roberts et al. (1998) [115]</td>
</tr>
<tr>
<td>Human, n = 30: 13 (age 18–36), 17 (age 49–65)</td>
<td>Uniaxial tensile</td>
<td>Tensile modulus: 16.9 ± 8.8 MPa; 12.2 ± 1.3 MPa (age 18–36); 20.5 ± 1.8 MPa (age 49–65)</td>
<td>Safshekan et al. (2016–2017) [116] [117]</td>
</tr>
<tr>
<td>Porcine, n = 1, age 1.5</td>
<td>Circumferential compression</td>
<td>Compression modulus: 1.78 ± 0.51 MPa (circumferential)</td>
<td>Wang et al. (2011) [118]</td>
</tr>
<tr>
<td>Porcine, n = 12</td>
<td>Uniaxial tensile; Circumferential &amp; Longitudinal compression</td>
<td>Tensile modulus: 1.30 ± 0.28 MPa; Compression modulus: 5.6 ± 2.0 MPa (circumferential); 1.1 ± 0.7 MPa (longitudinal)</td>
<td>Hoffman et al. (2016) [119]</td>
</tr>
<tr>
<td>Rabbit, n = 3, age 3 months</td>
<td>Uniaxial tensile</td>
<td>Tensile strength: 0.85 ± 0.28 MPa; Tensile modulus: 0.20 ± 0.07 MPa</td>
<td>Jung et al. (2016) [31]</td>
</tr>
<tr>
<td>Rabbit, n = 3</td>
<td>Circumferential compression</td>
<td>Compression modulus: 1.4 ± 0.5 MPa</td>
<td>Hung et al. (2016) [120]</td>
</tr>
</tbody>
</table>

1.4.4.2 Mechanical properties of scaffolds

Intrinsic mechanical properties of trachea, as an organ that can withstand pressure variations, shall prevent its collapse during respiration [121]. Challenges regarding the physical properties can thus be addressed when choosing a biomaterial for its replacement. The biomaterial that is intended to emulate a natural trachea must be strong enough to withstand such pressure without collapsing, whilst being soft enough to avoid damaging the blood vessels when rubbing against them [122]. In fact, it is reported that mechanical properties of most studied materials were significantly inferior to native tracheal cartilage [108]. As a comparison to mechanical
properties of native trachea listed in Table 1.1, *in vivo* mechanical properties of scaffold materials and constructs in TTE for porcine and rabbit are shown in Table 1.2. As it can be seen, scaffolds intended for *in vivo* preclinic applications in porcine and rabbit showed comparative or even superior mechanical properties compared with native trachea, preventing airway from collapsing.

**Table 1.2** Mechanical properties of materials and constructs in TTE (porcine and rabbit)

<table>
<thead>
<tr>
<th>Target</th>
<th>Material</th>
<th>Test type</th>
<th>Reported constants</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine, n = 12</td>
<td>Decellularized trachea from porcine</td>
<td>Uniaxial Tensile</td>
<td>Maximum force = 171 ± 5 N, rupture force = 56 ± 2 N; strain at break = 200 ± 8%</td>
<td>no significant differences were found in their strain ability compared with native tracheas</td>
<td>Jungebluth et al. (2009) [123]</td>
</tr>
<tr>
<td>Porcine, n = 5</td>
<td>Decellularized trachea from porcine</td>
<td>Uniaxial Tensile</td>
<td>Maximum force = 175 ± 8 N, rupture force = 56 ± 2 N; strain at break = 198 ± 7%</td>
<td>pigs were alive with no signs of airway collapse</td>
<td>Go et al. (2010) [124]</td>
</tr>
<tr>
<td>Rabbit, n = 9</td>
<td>Decellularized trachea from rabbit</td>
<td>Circumferential compression</td>
<td>Compression modulus = 1.54 ± 0.66 MPa</td>
<td>mechanical properties did not differ significantly from the fresh native trachea</td>
<td>Hung et al. (2016) [120]</td>
</tr>
<tr>
<td>Rabbit, n = 32</td>
<td>PU (3D printed)</td>
<td>Uniaxial tensile</td>
<td>Tensile strength = 3.21 ± 1.02 MPa, Tensile modulus = 2.81 ± 0.58 MPa</td>
<td>biomechanical properties were continually maintained for 16 week-period</td>
<td>Jung et al. (2016) [31]</td>
</tr>
<tr>
<td>Rabbit, n = 4</td>
<td>PCL (3D printed)</td>
<td>Uniaxial tensile &amp; Circumferential compression</td>
<td>Tensile strength = 0.32 ± 0.08 MPa; Compressive strength = 0.06 ± 0.01 MPa</td>
<td>scaffold showed superior mechanical properties</td>
<td>Park et al. (2018) [125]</td>
</tr>
</tbody>
</table>

*Note*: PCL, polycaprolactone; PU, polyurethane; porcine trachea resembles that of human, and the rabbit model is similar to that of the human infant.

However, stenosis may still present a serious problem even in cases where mechanical strength is sufficient [126, 127]. As mechanical stress changes, living tissue reshapess throughout life. As responding to dynamic mechanical signals, cellular and ECM stiffness exhibit responsive changes, which play key roles in a variety of physiological and pathological processes, including cell movement [128, 129], migration [130, 131], proliferation [129, 132],.
differentiation [133, 134] and vascular disease [135]. Clinically available scaffold materials and constructs are manufactured from a limited library of biological materials. Though a long-term record of mechanical stability and passive structural support has been established, these materials are generally stronger and stiffer than surrounding tissue and have little adaptability to biological and physical stimuli. Particularly, the mechanical mismatch at the interface between the stent and the native tissue determines the long-term integration of the implant [136, 137], which can stimulate tissue remodelling, leading to poor clinical outcomes such as stress shielding, extrusion of implants or organs [137, 138].

1.4.4.3 Cell response to scaffolds with different mechanical properties

Normal tissue cells are expected to be anchorage dependent, where a solid possessing certain stiffness is needed for cell adhesion [139]. ECM stiffness in microenvironment is capable of modulating stem cell lineage [109], and serves as a determinant in cellular phenotype [140]. For example, a hard matrix (>100 kPa) can differentiate stem cells into the osteogenic lineage, and moderate stiffness (10~100 kPa) affects the commitment of cells to the myogenic lineage, while stem cells towards neuronal lineage can be directed by soft matrix (< 10 kPa) [141]. Darnell et al. proved that matrix stress relaxation can mediate scaffold remodeling and thus tissue formation by implanting alginate gels with different stress relaxation rates carrying human mesenchymal stem cells into rat calvarial defects [142].

Tissue engineering scaffolds or implants can be considered ECM analogs, so in addition to providing structural support, the desired cellular behavior should be guided within a given geometric framework [143]. Optimizing the biomechanical properties of scaffolds, surface chemistry and microstructure are critical to imparting optimal cell behavior [136]. The challenges for developing both structural and functional scaffolds still remain: air-sealing capabilities, the delay in vascularization leading to necrosis, re-epithelialization or mechanical mismatch are the major problems [144]. Various responsive polymeric materials have been developed regarding tissue engineering, drug delivery and medical devices.

1.4.5 3D printing in scaffold fabrication

As seen in the previous subsections, scaffolds play a pivotal part by serving as a substrate for cellular migration and subsequent tissue formation. Various methods have been developed to construct tissue engineering scaffolds, including gas foaming, freeze drying, particulate leaching, and thermally induced phase-separation (TIPS). Gas foaming [145] permits good interconnectivity of the pores but requires from the use of highly viscous monomers/solutions or foaming agents that may impact the biological response of the scaffold. Freeze-drying [146], and particulate leaching [147, 148], often used to fabricate porous membranes, can control the pore size to a certain extent; however, they can result in skin-effect, non-uniform porous structures, limited interconnectivity, isolated pores or tightly close geometric packing, which in turns can affect the cellular-scaffold interactions. TIPS [149, 150], on the other hand, can offer
improved control over the pore size, pore morphology, and pore interconnectivity by varying the processing conditions. However, these techniques share one general limitation, which is the lack of control over the required uniform aperture and size, especially in larger, complex configurations. Compared to conventional subtractive and formative manufacturing processes, 3D printing is the most suitable technique for making products with complex internal structures, digitally defined inter-connective pores size and pore structure at macro- and microscales, improved versatility and customization without tooling systems, and short lead time [151].

The variety of available 3D printing methods demonstrates the robustness of this approach in the tissue engineering domain. 3D printing can be broadly classified into light-based and ink-based printing. Comparison of some of the commonly-used methods is presented in Table 1.3.

Stereolithography (SLA) involves using ultraviolet light to solidify a liquid resin that consists of photosensitive substituents (i.e., monomer and photoinitiator). A thin layer of liquid resin is laid before it is exposed to ultraviolet light and photopolymerisation takes place to harden the polymer [152, 153]. The mechanical properties of the SLA are insufficient, the liquid resin in the final product is captured, and the most important is the lack of biocompatible and biodegradable materials for SLA, which restrict its comprehensive application in TTE.

Alternatively, fused deposition modelling (FDM) is one of the most widespread methods of fabricating devices and scaffolds for TE applications [154]. FDM is a process in which filaments are driven through an electrically heated nozzle that melts the polymer material and builds a 3D structure layer by layer before solidification at room temperature [155]. The materials in this context are commonly referred to as a filament and are described as thermoplastics because their flow is observable above the filaments’ melting point, with solidification becoming apparent upon cooling [156]. Examples of commonly thermoplastic polymers include PCL, PLA, and non-degradable polyurethanes (PUs). The versatility of this technique can be used to create scaffolds based on composite materials possessing multiple properties.

However, as for polymeric solutions, a common hurdle for direct printing is to shape solutions into appropriate scaffolds in which the porosity and interconnectivity of the network is difficult to control. By using 3D printing in combination with freeze-drying [157, 158], and indirect 3D printing negative moulding combined with phase separation [159-161], several methods have been used to make polymer suspensions, solutions or resin-based scaffolds.
Table 1.3 Characteristics of commonly-used 3D printing approaches (adapted from Du et al. (2018) [162])

<table>
<thead>
<tr>
<th>Type</th>
<th>Category</th>
<th>Principle</th>
<th>Appliance materials</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLA</td>
<td>Light-based</td>
<td>A single beam laser to polymerize or crosslink the polymer to get thin layers and then stacks the struts layer-by-layer</td>
<td>Photosensitive polymers</td>
<td>High resolution; Short production times; complex internal structures</td>
<td>High cost; inadequate mechanical property; toxic uncured resin; light-sensitive; low photon/chemical resistance; limited printable biomaterials</td>
</tr>
<tr>
<td>SLS</td>
<td>Light-based</td>
<td>A high-power laser for metal or ceramic powder sintering to form a scaffold layer-by-layer</td>
<td>Metal, ceramics (a fine powder form)</td>
<td>Support not required; process multiple materials in a single bed</td>
<td>Slow, bulky; expensive; rough surface</td>
</tr>
<tr>
<td>DIW</td>
<td>Ink-based</td>
<td>Materials are extruded through the nozzles by a compressed gas to form individual lines that solidify onto a build plate layer-by-layer</td>
<td>Viscous materials (inorganic/organic composite paste, hydrogels)</td>
<td>Low temperature; cells and bioactive molecules can be printed</td>
<td>Solvent required; easy to sag or collapse during printing</td>
</tr>
<tr>
<td>FDM</td>
<td>Ink-based</td>
<td>A filament of the desired material is fed and melted in a vessel by heat and extruded from the nozzle, and deposited layer-by-layer</td>
<td>Polymers or polymer composite materials or ceramic composite materials</td>
<td>Mechanical properties of the products close to those by conventional injection moulding/extrusion; solvent not required; low cost; good strength; multi-material capability</td>
<td>Need to be formed into a filament; high temperature; anisotropy; nozzle clogging; limited printable biomaterials</td>
</tr>
</tbody>
</table>

*Note:* SLA, Stereolithography; SLS, Selective laser sintering; DIW, Direct ink writing; FDM, Fused deposition modelling

### 1.4.6 Applications in tracheal tissue engineering

#### 1.4.6.1 Preclinical applications

Many researchers have explored the potential of using 3D printed external splint to treat trachea defects. However, this approach is still in the early developmental stages of alleviating the damage of long-segmented defects of the trachea, without greatly compromising the patient's life. 3D printing creates an avenue to develop versatile and specialised-engineered objects, making this technology ideal for building an anatomical model of the trachea. With the increasing progress of 3D printing, this method has begun to extend its application in TTE gradually. The ways in which 3D FDM printing can be applied *in vivo* for tissue engineering purposes in recent years is depicted in Table 1.4. Studies addressed in this table proved the application of the 3D printed scaffold in *in vivo* animal studies. Thus, featured by precise positioning of various biomaterials to fabricate tissues and organs, 3D FDM printing should be an ideal method to create customized airway replacement grafts for tracheal reconstruction.

Numerous preclinical experiments have been carried out to investigate feasibility of various synthetic polymers in trachea repairment, both *in vitro* and *in vivo*. Despite progressive development and application of these synthetic materials in animal studies, few clinical studies have been reported [43, 163]. Therefore, further studies are vital to prepare ideal materials that can be clinically trialled [49]. It is noteworthy to mention that all synthetic biomaterials should be critically evaluated in well-established clinical trials prior to its application in humans [164].
### Table 1.4 Summary of 3D FDM printing technique in TTE applications (porcine and rabbit in vivo)

<table>
<thead>
<tr>
<th>Target</th>
<th>Printing material</th>
<th>Outcome</th>
<th>Ref./Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>PCL</td>
<td>Successful reconstruction and respiratory mucosa, cilia regeneration, and successfully integrated with the adjacent trachea without disruption or granulation tissue formation.</td>
<td>Chang et al. (2014) [165]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>PLA</td>
<td>All animals survived with a well-mucosalized tracheal lumen and a newly formed cartilage in the region where the graft was present.</td>
<td>Goldstein et al. (2015) [166]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>PCL</td>
<td>Asymmetrically porous membrane reinforced by 3D printed mesh is promising as a 360-degree tracheal substitute with comparable survival and luminal patency.</td>
<td>Lee et al. (2016) [167]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>PU</td>
<td>The tracheal scaffolds were patent for 16 week-period, re-epithelialization and ingrowth of the connective tissue after 4 weeks and ciliated respiratory epithelium with ciliary beating after 8 weeks of implantation were observed.</td>
<td>Jung et al. (2016) [31]</td>
</tr>
<tr>
<td>Porcine</td>
<td>PCL</td>
<td>Graft was structurally intact and well-incorporated, respiratory mucosal coverage and vascularity of the graft was observed.</td>
<td>Rehmani et al. (2017) [168]</td>
</tr>
<tr>
<td>Rabbit</td>
<td>PCL</td>
<td>Mean survival time in Group I (culture with chondrocytes for 2 weeks) was 14 ± 5 days, and in Group II (culture with chondrocytes for 4 weeks) was 22 ± 16 days, with the longest survival time being 10 weeks in Group II.</td>
<td>Gao et al. (2017) [169]</td>
</tr>
</tbody>
</table>

**Note:** PCL, polycaprolactone; PLA, polylactic acid; PU, non-degradable polyurethane
1.4.6.2 Clinical applications

In recent years, there have been several reports on the use of TTE to treat long-segment airway defects. Table 1.5 provides a summary of the studies involving different cells, cell growth factors, and the scaffolds that are specific to human TTE within 2008–2018.

Table 1.5 Summary of human tracheal tissue engineering ranging 2008–2018 (adapted from Chiang T et al., 2016) [60].

<table>
<thead>
<tr>
<th>Ref./Year</th>
<th>Target</th>
<th>Cell source</th>
<th>Growth factor</th>
<th>Scaffold</th>
<th>Outcome</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macchiarini, et al. (2008) [170]</td>
<td>30-year-old female</td>
<td>hBEpiCs, chondrocyte, BM-MNCs</td>
<td>TGF-β3,</td>
<td>Decellularized cadaveric trachea</td>
<td>Complete epithelial layer with normal mucociliary clearance</td>
<td>4 months</td>
</tr>
<tr>
<td>Omori, et al. (2008) [171]</td>
<td>71-year-old male</td>
<td>Venous blood</td>
<td>None</td>
<td>Marlex mesh patch with spiral rings covered by collagen sponge</td>
<td>Small leak treated with drainage, epithelialization (22 months)</td>
<td>22 months</td>
</tr>
<tr>
<td></td>
<td>59-year-old female</td>
<td>Venous blood</td>
<td>None</td>
<td>Marlex mesh patch with spiral rings covered by collagen sponge</td>
<td>Complete epithelialization (11 months)</td>
<td>12 months</td>
</tr>
<tr>
<td>Kanemaru et al. (2010) [172]</td>
<td>39-, and 71-year-old female</td>
<td>Venous blood</td>
<td>FGF</td>
<td>Marlex mesh patch with spiral rings covered by collagen sponge</td>
<td>Patents upper airway breathe easily</td>
<td>6 months</td>
</tr>
<tr>
<td>Delaere et al. (2010) [63]</td>
<td>55-year-old female</td>
<td>None (buccal mucosa used as patch)</td>
<td>None</td>
<td>Tracheal allograft into forearm, free flap</td>
<td>Complete mucosal (4 months), intact blood supply</td>
<td>1 year</td>
</tr>
<tr>
<td>Elliott et al. (2012) [54]</td>
<td>12-year-old male</td>
<td>BM-MNCs</td>
<td>TGF-β3</td>
<td>Decellularized cadaveric trachea</td>
<td>Malacia (6 weeks) requiring stenting, granulation, and retained secretions</td>
<td>42 months</td>
</tr>
<tr>
<td>Study Reference</td>
<td>Age</td>
<td>Gender</td>
<td>Cells Used</td>
<td>Growth Factor</td>
<td>Tissue Source</td>
<td>Epithelial Characteristics</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----</td>
<td>--------</td>
<td>------------</td>
<td>---------------</td>
<td>---------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Gonfotti et al. (2014) [173]</td>
<td>30-year-old female</td>
<td>hBEpiCs, chondrocytes, BM-MNCs</td>
<td>TGF-β3</td>
<td>Decellularized cadaveric trachea</td>
<td>Epithelium with a continuous layer of basal membrane and small blood vessels</td>
<td>5 years</td>
</tr>
<tr>
<td>Hamilton et al. (2015) [174]</td>
<td>12-year-old male</td>
<td>BM-MNCs</td>
<td>TGF-β3</td>
<td>Decellularized cadaveric trachea</td>
<td>Complete mucosal lining at 15 months, differentiated respiratory layer and no abnormal immune activity</td>
<td>4 years</td>
</tr>
<tr>
<td>Steink e et al. (2015) [175]</td>
<td>26-year-old male</td>
<td>Microvascular endothelial and skeletal muscle cells</td>
<td>None</td>
<td>Decellularized porcine jejenum (used as patch)</td>
<td>Complete incorporation into surrounding tissue (2.5 years)</td>
<td>2.5 years</td>
</tr>
<tr>
<td>Elliott et al. (2017) [176]</td>
<td>15-year-old female</td>
<td>hBM-MSCs, hBEpiCs</td>
<td>TGF-β3</td>
<td>Decellularized tissue-engineered tracheal graft</td>
<td>Deceased</td>
<td>21 days</td>
</tr>
</tbody>
</table>

Note: hBM-MNCs, human bone marrow-derived mononuclear cells; hBEpiCs, human bronchial epithelial cells; TGF-β3, transforming growth factor beta 3; FGF, fibroblast growth factor; hBM-MSCs, human bone-marrow mesenchymal stem cells.

Complete establishment of tracheal re-epithelialization plays a particularly important part in the long-term success of TTE. Numerous studies in TTE have provided encouraging results in culturing and proliferating tracheal epithelial cells in vitro and transplanting these cells in vivo in an appropriate manner, while the clinical application of human tracheal epithelial cells remains limited. Omori et al. [171, 177] used in situ tissue engineering techniques to repair the human larynx and trachea. They reported that the man-made material was covered by epithelial growth and the epithelial formation continues to completely cover the man-made material. In 2008, the entire airway was replaced for clinical use [170]. The epithelial cells were seeded on the decellularized tracheal graft and then cultured in a bioreactor for 96 hours. After transplantation, the graft was completely covered by mucosa after 1 month and reached mucociliary clearance at 6 months. In addition, according to a 5-year follow-up study [173], it
was finally confirmed that there were pseudostratified ciliated columnar epithelial cells with a continuous basement membrane.

There are many potential complications to consider when using tissue engineered tracheal in a pediatric population compared to adults. The first paediatric TTE recipient of a 12-year-old boy was documented in 2012, whereby the patient suffered from a congenital tracheal stenosis and pulmonary sling [54]. Prior to the TTE, the donor trachea was decellularized for 3 days and seeded intra-operatively with BM-MSC tracheal mucosa. Transplant revascularization was performed within 1 week after transplantation, but cytological evidence of epithelial cell recovery was not evident until 1 year. Thereafter, a 4-year follow-up study was reported [178], which showed complete mucosal lining at 15 months, histological cytology showing differentiated respiratory layers and no abnormal immune activity. In a more recent case, a 15-year-old girl was given a novel tracheal graft that had undergone decellularization and was seeded with stem cells following from failed attempts to treat trachea stenosis [176]. Early results following its application were encouraging, but the occurrence of an acute event, i.e., intrathoracic bleed that blocked her airway led to the untimely death of the patient. The unanticipated outcome underpins the inherent challenges of preclinical in vivo models; one cannot anticipate all complex clinical scenarios before the implantation of a graft.

Vascularization of the tracheal tissue is essential to achieve a successful outcome for adequate supply of nutrients and oxygen to the luminal mucosal lining. Tissue engineered scaffolds must rapidly develop their own blood supply to support cellular metabolism, which is established by penetrating small blood vessels between the cartilage rings [34]. Considering the importance of revascularization for tracheal epithelial regeneration and survival, some studies have been carried out to investigate the safe recovery of blood supply. Delaere et al. [179, 180] performed a series of tracheal allografts using heterotopic vascularized tracheal allografts after abscission of immunsuppressive therapy. Allogeneic grafts are fully vascularized and have a rich blood supply prior to inoculation of tracheal allografts with recipient epithelial cells. A rapid and stable formation of the epithelial layer was observed after transplantation. In addition, a bioartificial vascular stent (BioVaSc) for tissue engineering was generated by Walles and colleagues [181, 182], in order to provide vascular anastomoses to supply bioengineered trachea after transplantation in vitro and in vivo. The BioVaSc thereafter expanded its clinical applications by inoculating autologous respiratory epithelial cells, endothelial progenitor cells, muscle and fibroblasts, where cellular components of the tracheal tissue were found in the vascularized tissue. The results showed that the vascular network of the graft was associated with the blood supply to the recipient after 12 weeks, and the bioartificial blood vessels were covered by the autologous ciliary airway epithelium.

Unfortunately, past tracheal replacement surgeries performed by Paolo Machiarrini during the period 2011-2012 resulted in high morbidity and mortality. The surgeries were performed using tissue engineered scaffolds that were seeded with the patient’s bone marrow cells and treated
with growth factors prior to transplantation. There are several aspects of the studies [183,281] that point to ethical misconduct. The primary concern is that although these scaffolds had undergone toxicity and biocompatibility testing in vitro, they had not been subjected to pre-clinical animal testing prior to their implantation in humans. Furthermore, lack of informed consent, inaccurate reporting of bronchoscope/histological results post-transplantation, as well as inadequate continuity of post-surgical care, all call to question the validity of these studies [183,282].

While there are achievements in the clinical field that show the positive impact of tissue engineering strategies on regenerative medicine in recent years, the downside is that researchers need to continue to critically evaluate outcomes, especially in terms of re-epithelialization and revascularization. Challenges associated with epithelial formation include optimizing cell culture conditions and migration of biological material, maintaining differentiated cell types, minimizing immune responses, and improving survival and quality of epithelial cells after transplantation [13]. In addition, restoration of blood supply is also among the greatest challenges in tracheal replacement [34]. In future studies, improved protocol of preclinical animal studies is needed to better understand the transplanted scaffold when in application.

1.5 Conclusion

Immense effort is continually invested in effectively replacing damaged organs, though several foreseeable challenges are exposed to us, i.e. epithelialization and re-vascularization of tracheal scaffolds. Various research groups around the world have used different approaches towards soft tissue reconstruction regarding these challenges, including tissue engineering and 3D printing. Due to the advancements in regenerative medicine research, there is scope for improvement to tissue engineering applications through 3D printing. Improvement of the cell seeding process on a microscopic level could lead to better functionality of tracheal constructs and also insight for larynx or lung regenerative care.

From a general perspective, transplantation of trachea is still a challenging procedure and no ideal solution has been discovered so far. Due to the adverse effects of lifelong immunosuppression, allogenic transplantation could not maintain its clinical feasibility and tissue engineered tracheal grafts have become the current promising therapeutic alternative. Despite encouraging research, the mechanism of tracheal tissue regeneration and host response to seeded cells is not fully understood and thus, tissue engineered tracheal grafts is still far from any routine clinical application. Further experimental and clinical researches have to be performed to improve current results and broaden the clinical application of airway tissue engineering.
Chapter 2

General Materials and Methods

2.1 Introduction

This chapter describes general materials and methods that were used in proceeding experimental chapters, including synthesis and fabrication of poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) material scaffold, characterization of the scaffold properties, in vitro and in vivo studies of cell culture on scaffold. Specific routines for various application scenarios will be elaborated in each following chapter. For example, materials and methods on cell culture of differentiated human bone-marrow derived mesenchymal stem cells (hBM-MSCs) and differentiated human bronchial epithelial cells (hBEpiCs) were described in section Chapter 4 and 5, respectively.

2.2 Synthesis and fabrication of PUU-POSS scaffold materials

2.2.1 Synthesis of PUU-POSS

Ethylenediamine and polyhedral oligomeric silsesquioxane (POSS) co-terminated poly (urea-urethane) (PUU) polymer solution was synthesized as needed, according to the methods described by Kannan et al. (2006) [184]. In brief, polycarbonate polyol, 2000 MW and trans-cyclohexanechlorotrimethylsilylsilsequioxane were placed in a 500 ml reaction flask equipped with mechanical stirrer and nitrogen inlet. The mixture was heated at 135°C in order to dissolve the POSS cage into the polyol and then cooled to 70°C. Flake 4,4’-methylenedianiline (phenyl isocyanate) (MDI) were added to the polyol blend and then reacted, under nitrogen, at 75°C~85°C for 90 min to form a pre-polymer. Dimethylacetamide (DMAC) was added slowly to the pre-polymer to form a solution. The solution was then cooled to 40°C. Chain extension of the pre-polymer was carried out by the drop wise addition of a mixture of ethylenediamine and diethylamine in DMAC to form an 18 wt.% solution of POSS modified poly (urea-urethane) (PUU) in DMAC. All chemicals and reagents were purchased from Sigma-Aldrich (Gillingham, UK).
2.2.2 Design and fabrication of 3D printing PVA preform

All polyvinyl alcohol (PVA) preforms for manufacturing and characterization of PUU-POSS scaffolds were designed in AutoCAD 2014 (Autodesk Inc., USA), exported as .stl files and transferred to Slic3r software (version 9.9) where they were sliced into an array of consecutive 200 μm layers. Two layers at both the bottom and top of the preform were kept solid, with all layers in between arranged in an organised internal scaffold pattern. The spacing between the scaffold frames was governed by the ‘infill density’, which was varied consecutively from 30% to 80% (with 10% incremental increases). The resulting G-code files were printed using PVA filament of diameter 1.75 mm by an Active X1 fusion deposition modeller (Active 3D Printers Ltd., UK) using Repetier-Host (version 0.56) software. The printed PVA ‘Preform shells’ were stored in an airtight desiccator to prevent any structural distortion due to absorption of atmospheric humidity.

2.2.3 3D printing confined thermal-induced phase separation (3D-TIPS) manufacturing polyurethane scaffolds

The polymer solution was injected into the PVA preform shell as a sacrificial mould through a surface puncture hole. Polymer filling resulted in a coloration effect within the shells, making it visible once the shell was completely filled. The filled shells were left to sit in a fume hood for 5 min, allowing for any air bubbles within the polymer to rise to the surface. A 3D-printed PVA preform method was used to confine thermal-induced phase separation (3D-TIPS) of PUU-POSS in the PVA shells at -20 ºC for 3 h. It was then slowly coagulated in water where the PVA subsequently dissolved.

2.3 Characterization of the scaffold material properties

2.3.1 Mechanical properties

2.3.1.1 Static mechanical tests

All machines were calibrated prior to use and all tests were performed at room temperature. Tensile rectangular wet-state bars (under deionized water) (preform size of 10 mm×56 mm×6 mm) were fabricated. Average thickness at the resolution was calculated from three measurements for each specimen. For tensile testing (Figure 2.1 A), samples (n=6) were subjected to uniaxial loads at 5 mm/min using an Instron 5655 tester (Instron Ltd., Norwood, MA, USA) and ultimate tensile strength, strain, strain at break and tensile modulus were obtained from data generated by Bluehill® software. Toughness was derived from the area under the stress-strain curve for each bar and then averaged for all samples. They were all collected with a stretching ratio of 50%.

Compression wet-state cuboids (under deionised water) of each scaffold group (preform size of 30 mm ×30 mm ×30 mm) were fabricated (using the equipment mentioned in Figure 2.1 B). Average thickness was calculated from three measurements for each specimen. Samples (n=6) were subjected to uniaxial loads at 5 mm/min using an Instron 5655 tester (Instron Ltd., Norwood, MA, USA), and the ultimate
CHAPTER 2 General Materials and Methods

compression strength, strain and compression modulus were obtained from data generated by Bluehill® software. They were all collected with a controlled strain (25%).

Figure 2.1 Instron 5565 tensile (A) and compression (B) testing machine equipped with a 500 N load cell.

2.3.1.2 Dynamic mechanical tests

Dynamic tensile mechanical properties of the scaffolds (preform size of 15 mm×6.5 mm×2 mm; \( n=2 \)) were tested at day 0 and after day 28 in a bioreactor at 37.5 °C in water using ElectroForce Biodynamic® Test Instrument 5160 (TA, USA) with 200 N load cells (Figure 2.2 A). The samples were loaded with a sinusoidal ramp of constant frequency of 1 Hz with a controlled strain (25%) for 200,200 cycles per sample.

Dynamic compression mechanical properties of the scaffolds (preform size of 10 mm ×10 mm ×10 mm; \( n=2 \)) were tested in compression modes at day 0 and after day 28, in a bioreactor at 37.5 °C in water using an ElectroForce Biodynamic® Test Instrument 5160 (TA, USA) with a 200N load cell (Figure 2.2 A). The samples were loaded with a sinusoidal ramp of constant frequency of 1 Hz with a controlled strain (25%) at increasing cycles up to 200,200 cycles per sample.

Temperature spectra of tensile and compression dynamic mechanical properties of the scaffolds were tested under frequency of 1 Hz and the strain of 1% for all samples by a dynamic mechanical analysis (DMA) instrument (TA Q800, USA) (Figure 2.2 B). The samples were cooled from ambient temperature to -80 °C and then held at this temperature for 10 min. The samples were then heated to 230 °C at a
heating rate of 2 °C/min according to the capacity of the instrument.

2.3.2 Thermal properties & crystallinity and phase identification

2.3.2.1 Thermal properties via DSC

Differential Scanning Calorimetry (DSC) is a thermal analysis technique that measures the temperature and heat flow accompanying with phase transitions in materials as a function of time and temperature in a controlled environment. These measurements provide quantitative and qualitative information about the physical and chemical changes that involve an endothermic or exothermic process or changes in heat capacity [185]. The scaffold was cut into around 10 mg in a standard aluminum pan. DSC samples were enclosed in a sample pan to avoid the direct contact between sample, furnace and sensor, different processing conditions were measured from -70 °C to 230 °C using DSC (Q1000, TA Instrument, USA) at a scan rate of 10°C/min (shown in Figure 2.3).
2.3.2.2 Crystalline structure of the scaffolds via XRD

The crystallinity and phase identification of the scaffold was analyzed using X-ray Diffraction (XRD Bruker D8 Advance, Germany) (shown in Figure 2.4), equipped with copper source and LynxEye™ silicon strip detector to record diffraction pattern of the scaffolds. The resulting XRD patterns were refined and the averaged size of crystallites was calculated by Total Pattern Analysis Solution (TOPAS, Germany) software. Preparation of scaffolds specimen for XRD material was carried out by pressing the powder in a standard sample holder by glass slide to make it at surface before placing the specimen holder in XRD scanning chamber. Data were collected from 5° to 100° (2θ) for 30 min in the step scan mode.

2.3.3 Characterization of porous structure and porosity measurement

2.3.3.1 Porosity via Mass Measurement

The porosity of PUU-POSS scaffolds was calculated using the equation below,
\[ \rho = (1 - \frac{d_a}{d_b}) \times 100\% \]  

(E2.1)

where the bulk density, \(d_b\), of PUU-POSS was taken as 1.15 g/cm\(^3\) (1150 kg/m\(^3\)), the apparent density, \(d_a\) (i.e., scaffold density), was calculated using the weight, \(W_a\) (g) and volume, \(V_a\) (cm\(^3\)) of each sample, \(d_a=W_a/V_a\). Six discs (of diameter 1.6 cm) were cut from each scaffold using a pre-shaped cutter.

### 2.3.3.2 Pore size, distribution and porosity analysis

A mercury intrusion porosimeter (Quantachrome Poremaster 60GT, UK) (Figure 2.5 A) was used to characterize the pore structure including the pore size, pore volume, size distribution and surface area of scaffolds. Freeze dried scaffolds with 9 mm in diameter, 4.5 mm in height, and the total mass less than 1.5 g were tested. The maximum applied pressure of mercury was 414 MPa. A wide range of pore diameters from 1100 micron down to 0.2 nm were tested. A representative scaffold pore volume-diameter curve was shown in **Figure 2.5 B**.

![Figure 2.5 PoreMaster Quantachrome](image)

**Figure 2.5** PoreMaster Quantachrome Poremaster 60GT (A); the scaffold pore volume-diameter curve (B).

### 2.3.4 Characterization of surface properties of the scaffolds

#### 2.3.4.1 Surface morphology via SEM

Scanning Electron Microscopy (SEM) was used for both structural and morphology analysis of coated samples. An ultra-high-performance field emission scanning electron microscope (Zeiss Supra 35VP FE-SEM, Germany) is shown in **Figure 2.6**. The field emission source and Gemini column provide with a high-resolution capability.
2.3.4.2 Structure at micro and nanoscale via TEM

The nanophase structure of sample was observed using high resolution field emission gun transmission electron microscope (FEG-TEM, JEOL2100, Japan). The TEM samples was embedded in an aromatic acrylic resin (LR White Embedding Medium, EMS, UK), and then cryo-micromtomed into a thin section using a CR-X cryosectioning system (RMC Boeckeler, USA) before stained by Ruthenium tetroxide (RuO$_4$) vapour (Figure 2.7).

Figure 2.7 Schematic illustration of TEM sample preparation procedure.
2.3.4.3 Surface chemical composition via EDX

Scanning electron microscopy (CrossBeam XB 1540 FIB-SEM; Carl Zeiss, Germany) with an Energy Dispersive X-Ray Spectroscopy (EDX) detector (EDAX Inc, USA) was used to analyze elemental surface regions of composition of the scaffolds (n=3) for detection of calcium (Ca) and phosphorous (P), as well as the relative distribution of these elements.

2.3.4.4 Wettability via captive bubble contact angle measurement

The captive bubble contact angle measurement (Figure 2.8 B) was used a DSA100 instrument (KRÜSS, Germany) (Figure 2.8 A), in conjunction with axisymmetric drop shape analysis-profile (ADSA-P) in sterile deionized water. Measurements were taken for each of the prepared surfaces. At this point, the angle the liquid made upon contact of air bubble with the polymer was measured using a circular algorithm technique, implemented in the KRÜSS drop shape analysis software. Values >90°θ are considered hydrophilic, and values <90°θ are considered hydrophobic in captive bubble technique [186] (Figure 2.8 C).

A KRÜSS DSA 100 (KRÜSS GmbH, Germany) system was used for static water contact angle measurements of samples (n=20), using a captive bubble sessile drop method. Sterile deionized water (diH2O) was used as a solvent, with a droplet volume of 3 μl. One droplet per sample and 20 samples (5 mm in diameter) were analyzed.

Figure 2.8 The KRÜSS contact angle apparatus (A): a captive bubble sessile drop measurement (B); captive bubble surface to measure the contact angles shape relating θ with hydrophilic/hydrophobic relationship (C).

2.3.4.5 Protein adsorption evaluation via BCA assay

Protein adsorption of polymer samples were assessed using the bicinchoninic acid assay (BCA) protein assay kit (Pierce™ thermo scientific, Rockford, IL, USA), following the manufacturer protocol. Briefly, all samples were prewetted in 70% ethanol for 30 min and rinsed (×3) with phosphate-buffered saline (PBS) overnight under gentle shaking (25 rmp). All samples (n=4 replicates) were immersed in 1.5 ml sterile PBS supplemented with 20% fetal bovine serum proteins (FBS) (Sigma, UK) for specific incubation
periods at 37 °C. After each incubation period, samples were washed (×4) with PBS, transferred into a fresh 24 well plates. To retrieve adherent proteins, samples were immersed in 2 ml of a 1% sodium dodecyl sulphate (SDS) (Sigma Aldrich, UK) solution in pure water. The well plate was incubated dynamically at room temperature for 1 h, after which 800 μl working reagents was added to each sample (100 μl) in a 96 well plates and incubated at 37 °C for 30 min. The absorbance was then read on a colorimetric plate reader (Anthos 2020 microplate reader, Biochrome Ltd, UK) at 562 nm. Standard curves were obtained from a series of dilutions of known standard bovine serum albumin (BSA) concentrations (total protein control). The data was normalised to % adsorption using the equation below:

\[
\text{% adsorption} = \frac{P_{\text{sample}}}{P_{\text{total protein}}} \times 100 \% \quad (E2.2)
\]

where \( P_{\text{total protein}} \) was the absorbance of standard bovine serum albumin (BSA) concentrations of total protein, \( P_{\text{sample}} \) was the absorbance of sample.

2.4 In vitro studies of cell culture on 3D scaffolds

2.4.1 Cell culture

2.4.1.1 Primary human dermal fibroblast cells (HDFs)

Primary human dermal fibroblast (HDF) cells derived from the dermis of normal human neonatal foreskin or adult skin (Culture Collections, HDF (106-05a) UK) were cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (50 μg/ml streptomycin, 50 μg/ml penicillin) solutions, and incubated at 37.5°C. Polymer discs (diameter of 11 mm and thickness of 3 mm) were cut from the 50% cuboids fabricated PUU-POSS scaffolds. All discs (\( n = 7 \)) were sterilised in 70% ethanol and stirred for 30 mins, after dried by air in a sterile cell culture hood, and then finally washed four times in sterile PBS. All discs placed in 48 well plates were pre-incubated in 500 μl of supplemented culture media for 24 h overnight. Each polymer disc was seeded with cells at a density of 2.5×10⁴ cells/scaffold (9×10⁴ cells/cm³) in 500 μl of cell culture medium. Wells containing 15 mm Thermonax coverslips (TCP) (Nunc, USA) were used as TCP positive controls.

2.4.1.2 Human bone-marrow derived mesenchymal stem cells (hBM-MSCs)

Human bone-marrow derived mesenchymal stem cells (hBM-MSCs) were subcultured and expanded with mesenchymal stem cell medium (MSCM; Sciencell™, California, USA) in a T75 flask. Before seeding, hBM-MSCs were washed with PBS and trypsinized with 3 ml of 0.25% trypsin-EDTA (Life-technologies, Paisley, UK) after PBS being aspirated. Cells were incubated at 37°C for 3 min and dislodged with gentle flask-tapping. Trypsin was neutralized with 7 ml of fresh MSCM and the cell suspension was transferred to a 15 ml tube; spun at 1,200 rpm for 5 min. After centrifugation, the concentrated cell pellet was resuspended in 2 ml of fresh MSCM and a viable cell count was performed by means of a Trypan Blue exclusion assay and a haemocytometer chamber.
Polymer discs (11 mm diameter and 3 mm thickness) were cut from the fabricated PUU-POSS scaffolds with 50% infill density. Samples \( (n=7 \text{ replicates}) \) were sterilised in 70% ethanol and stirred for 30 minutes before being washed four times in sterile PBS. All discs placed in 48-well plates were pre-incubated in 500 µl of MSCM for 24 hrs overnight. Each scaffold was then seeded at a density of \( 9 \times 10^4 \text{ cells/cm}^3 \), corresponding \( 2.5 \times 10^4 \text{ cells/scaffold (P2)} \) in 500 µl of MSCM based on a preliminary 10-day study evaluating metabolic activity of the scaffolds at different cell-seeding densities. Media was replaced every three days, and the metabolic activity of cells was monitored on days 1, 3, 7, 10 and 14 to determine cell viability through alamarBlue® (AB) (Serotec Ltd, Kidlington, Oxford, UK) testing. At each day point, total DNA content was also quantified using a fluorescent Hoechst 33258 stain. Tissue culture plate (TCP) was used as comparison.

2.4.1.3 Human bronchial epithelial cells (hBEpiCs)

The human bronchial epithelial cell line (hBEpiCs) (ScienCell, California, USA) was used with BEpiCM media (ScienCell, California, USA) in a T75 flask. Before seeding, cells were washed with PBS and trypsinized with 3 ml of 0.25% trypsin-EDTA (Life-technologies, Paisley, UK) after PBS being aspirated. Cells were incubated at 37°C for 3 min and dislodged with gentle flask-tapping. Trypsin was neutralized with 7 ml of fresh BEpiCM and the cell suspension was transferred to a 15 ml tube; spun at 1,200 rpm for 5 min. After centrifugation, the concentrated cell pellet was resuspended in 2 ml of fresh BEpiCM and a viable cell count was performed by means of a Trypan Blue exclusion assay and a haemocytometer chamber. Cells were cultured at 37.5 °C and 5% CO\(_2\) in a humidified atmosphere. Cells were used in passage 5\(^{th}\) and Scaffolds were cut in to 16 mm diameter discs and clipped to CellCrown™ polycarbonate transwell housing for 24-well plate (Scaffdexoy, Tampere, Finland). A number of \( 5 \times 10^4 \text{ cells (2.5} \times 10^5 \text{ cells/cm}^3) \) were plated on to 3D scaffolds.

2.4.1.4 Mouse embryonic dermal fibroblast cells (3T3-J2 cells)

Mouse embryonic dermal fibroblasts (3T3-J2 cells; Howard Green lab, Harvard University, 3T3-J2 CVCL_W667, USA) were cultured on tissue culture plastic in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic (50 µg/ml streptomycin, 50µg/ml penicillin) solutions, and incubated at 37.5°C. Polymer discs (11 mm diameter and 1.5 mm thickness, \( n=4 \text{ per group} \) were cut and sterilized in 70% ethanol and stirred for 30 minutes, air-dried in a sterile cell culture hood and finally washed in sterile phosphate-buffered saline (PBS). Discs were placed in 48-well plates and pre-incubated in 500 µl of culture media for 24 h overnight.

Scaffolds were seeded with third-passage (P3) cells at a density of \( 9 \times 10^4 \text{ cells/cm}^3 \) (1.3\times10^4 \text{ cells/scaffold}) in 500 µl of cell culture medium in 48 wells. Media was replaced every three days, and metabolic activity was monitored on days 1, 3, 7, and 14 by the alamarBlue® (AB) assay (Serotec Ltd, Kidlington, Oxford, UK) as per manufacturer’s instructions [187]. Total DNA content was also quantified at each time point using a fluorescent Hoechst 33258 stain [188].
2.4.2 Optimisation of cell seeding density on scaffolds

Primary human dermal fibroblast (HDF) cells (Culture Collections, HDF (106-05a) UK) were cultured in Dulbecco’s modified Eagles medium (DMEM) (Gibco, Life Technologies, UK), supplemented with 10% foetal bovine serum (FBS) (Gibco, Life Technologies, UK) and 1% antibiotic (50μg/mL streptomycin, 50μg/mL penicillin) solutions (Gibco, Life Technologies, UK), and incubated at 37.5°C.

Polymer discs (diameter 11mm, thickness 3 mm) were cut from the 50% cuboids fabricated from each of the three PUU-POSS scaffold conditions. Discs (n=7) were sterilised in 70% ethanol and stirred for 30 minutes, followed by air drying in a sterile cell culture hood, before finally being washed four times in sterile phosphate-buffered saline (PBS). All discs were placed in 48 well Corning culture non-treatment plates (VWR International, UK), pre-incubated in 500µl of supplemented culture media for 24 h. Cells suspensions from passage 4th (P4) at five different densities (3× 10^4, 9× 10^4, 27× 10^4, 81× 10^4, and 243× 10^4 cells/cm³; corresponding respectively to 0.85× 10^4, 2.5× 10^4, 7.7× 10^4, 23× 10^4, and 69× 10^4 cells/scaffold) were then seeded onto each scaffold and cultured under standard culture conditions of 5% CO₂ in incubator at 37°C with medium renewal every 2~3 days. The culture media used was Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Life Technologies, UK), supplemented with 1% penicillin-streptomycin solution (Gibco, Life Technologies, UK) and 10% FBS solution (Gibco, Life Technologies, UK). One day after seeding, scaffolds were transferred to a new 48-well plate to avoid false positive readings from cells adhering to the tissue culture plastic rather than the scaffold. Cell viability and proliferation were assessed using an alamarBlue® assay (Serotec Ltd, Kidlington, Oxford, UK) performed according to manufacturer's instructions, as reported by Molna et al. (2010) [187].

2.4.3 Cell metabolic activity via Alamar Blue® (AB) assay

The metabolic activity of cells was monitored to determine cellular viability using the commercially available alamarBlue® assay (Serotec Ltd, Kidlington, Oxford, UK), as previously described. Briefly, 10% (v/v) of AB (100 µl/ml solution of AB in DMEM) was added to the culture media of the cell-seeded polymer discs 4 h prior to the end of cell culture. Aliquots (100 µl) were placed in a 96 well plate, and the absorbance read on a fluorescence plate reader (Fluroskan Ascent FL reader, Thermo Labsystems, Basingstoke, UK) at 530 nm excitation wavelength and 620 nm emission wavelength, respectively. The alamarBlue® reading in cell culture medium of TCP/polymer scaffold without cell seeded was used as a negative control.

\[
\text{% Relative Fluorescence (alamarBlue®)} = \frac{F_{\text{test}} - F_{\text{control}}}{F_{100\% \text{relative fluorescence}} - F_{\text{control}}} \times 100 \% \quad (E2.3)
\]

where F_{control} was the fluorescence from alamarBlue® in media without cells, F_{test} was fluorescence from samples and F_{100\% \text{relative fluorescence}} was a complete reduction of alamarBlue® generated by autoclaving alamarBlue® in media (according to company’s instruction).
2.4.4 DNA content quantification via Total DNA Hoechst staining

The total DNA contents of cultured cell were determined using fluorescent Hoechst 33258 stain as previously described by Rago et al. (1990) [188]. Briefly, cells were lysed (×5) using freeze-thaw cycles following the removal of cell culture supernatants stored froze. Lysed cells were stored in a freezer at -80°C and thawed to room temperature. The lysate was incubated with molecular biology grade water (Sigma, UK) for 1 h in each cycle and Hoechst stain (Benzamide 33258, Sigma, UK) was added at a final concentration of 2 μg/ml. The fluorescence of Hoechst dye was measured at 360 nm excitation wavelength and 460 nm emission wavelength using a fluorescence microplate reader (Fluoroskan Ascent FL, Thermo Labsystems, UK). Total DNA and cell number standard curves were obtained from serial dilutions of known DNA concentrations (positive control) (calf thymus, Sigma, UK) and different cell densities, respectively. The DNA standard curve was generated using standard calf thymus DNA provided with the kit.

2.4.5 Total extracellular collagen production quantification

The amount of extracellular acid-soluble collagen (types I - V) was measured in cells cultured on 3D scaffolds. Cells were removed from each scaffold by trypsinization, centrifuged at 1200 rpm for 5 min with removal of supernatant and resuspension in 0.1% of 0.5 M acetic acid, followed by three rinses in PBS. Samples (n=4) were allowed to solubilize overnight. The quantity of acid soluble collagen per sample in the extraction solution was measured using a Sircol™ assay (Biocolor, UK), which labels the soluble collagen molecules with 0.1% Picro Sirus Red (PSR) solution (Sigma Aldrich, UK) overnight at 5 °C to form an insoluble complex. The dye contains a reagent that specifically binds to collagen. Acid-soluble collagen, 100 μl per sample, was added to 500 μl of dye binding reagent and incubated at 37.5°C, 5% CO₂ for 1 h to form the soluble dye-collagen complex solution. Dye-bound collagen was removed by centrifugation and the dye was then solubilized in alkaline. The absorbance of the resulting mixture was read at 540 nm on an absorbance plate reader (Anthos 2020 microplate reader, Biochrome Ltd, UK). The concentration of soluble collagen per sample was calculated from a standard curve using bovine collagen standards kit (n=6). Results were normalized to the amount of collagen (μg/ml) in each sample.

2.4.6 Assessment of sulfated glycosaminoglycan (sGAG) synthesis

Sulfated glycosaminoglycans are among one of the main extracellular matrix (ECM) components. Cells were cultured on 3D scaffolds at different time points, and the ratio of sGAGs/DNA was quantified over a 10-day period by means of a Blyscan™ sulphated glycosaminoglycan assay (Biocolor Ltd., Antrim, UK).

At each day point, samples of each group (n=4) were collected to determine sGAG content in the matrix, and total DNA levels were measured as well for normalization purposes. Then 0.5 ml of 0.25% trypsin-EDTA (Life-technologies, Paisley, UK) were added into each scaffold well. Extracted cell suspensions of 250 μl and 1 ml of Blyscan dye reagent were transferred to micro-centrifuge tubes, which were placed on a gentle mechanical shaker for 30 min. During this period, sGAG-dye complex would form and
precipitate out from the soluble unbound dye. The tubes were later spun at 12,000 rpm for 10 min and the supernatant was carefully removed. Later 0.5 ml of dissociation reagent was added to the tube with interval mixing in a vortex to help resuspend the sGAG-dye complex. Finally, 200 µl of each suspension was transferred to a clear 96-well plate and the absorbance was read at 630 nm using a microplate reader (Biotek, Swindon, UK), calculated using a standard curve generated from glycosaminoglycan standards provided with the kit.

### 2.4.7 Live-Dead cell survival assay and immunohistochemistry

Cell survival on the various PUU-POSS scaffolds was measured using a Live/Dead fluorescence assay (Invitrogen) as per manufacturer guidelines. Live cells stained by uniformly green-fluorescent calcein-AM stained and non-viable/dead cells stained by pyknotic red-fluorescent ethidium homodimer-1 cells were visualised using a confocal microscope (Leica TCS SP8vis, Germany) at ×10 and ×20 water immersion objective lens (Figure 2.9).

Figure 2.9 Schematics of LIVE/DEAD ® Viability/Cytotoxicity Kit for cell culture, expansion by Leica TCS SP8vis confocal microscope.

Cells cytoskeleton and attachment to the various PUU-POSS scaffolds were studied using Fluorescein Isothiocyanate Labeled (FITC)-labelled phalloidin (Life-technologies, Paisley, UK) according to the manufacturer’s instructions. Briefly, cell-laden polymer discs were harvested, first fixed with 4% paraformaldehyde (PFA, Muto Pure Chemicals, Sigma, UK) for 12 h at 4°C and rinsed three times with PBS. They were permeabilised with 0.1% Triton-X 100 for 15 mins and rinsed three times with PBS. In order to avoid non-specific binding, cell-laden polymer samples were blocked with 1% bovine serum albumin (BSA) solution for 30 mins and rinsed. The cells were stained with FITC-phalloidin, and then the nuclei counterstained using a blue-fluorescent Hoechst 3334244 (Sigma Aldrich, UK). Images were taken using a confocal microscope (Leica TCS SP8vis, Germany) with ×10 and ×20 water immersion objective lens. The Z-stacking images were acquired by scanning 9-point areas (each point areas sized
1.16 mm × 1.16 mm) throughout 3 mm thickness of the scaffold at 7 µm/Z-step. Image stacks were visualized and analysed using ImageJ software (NIH, USA).

2.4.8 Cell morphology via SEM scaffolds preparation

**Figure 2.10** Schematic illustration of SEM cell sample preparation: cells on the scaffolds were fixed in SEM fixative (PFA and glutaraldehyde), post-fixed in osmium tetroxide and uranyl acetate for later dehydration in various ethanol solutions.

Cells on the polymer scaffolds were stored in a vial containing primary fixative solution, 4% PFA and 2% glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.4 and 4 °C overnight. Samples were washed thereafter three times with PBS and then three times with bi-distilled water so as the fixatives’ residues could be completely removed. Gradually, water was replaced with an intermediate medium (ethanol) starting from a concentration of 10~50% (v/v). At this time, the incubation time was 10 min for each solution. Samples were then infiltrated in sequence with a solution of 70% (v/v) ethanol for 10 min; 90%, and 100% (v/v) ethanol were exchanged three times for 10 min (each step). In the end, samples were stored in 0.1 M propylene oxide 10 min in a sealed dish. This method is depicted and summarized in Figure 2.10.

Based on the method of critical point drying (CPD) upon CO₂ phase transition, samples were transferred into the chamber of a CPD machine (CPD 030, BAL-TEC, Schalksmuehle, Germany) so as to ensure that the cells were continuously immersed in 100% ethanol. The system was slowly cooled down to 10 °C (typically 1°C/step). The ethanol was gradually replaced with CO₂ liquid. This consist of typically
10 ethanol/CO\textsubscript{2} exchange repetitions. Then, the chamber’s temperature was increased by 1 °C/step until reaching a temperature of about 40 °C. At the critical temperature and pressure, the CO\textsubscript{2} liquid turns into the supercritical state. The samples in the chamber completely dried off after the CO\textsubscript{2} gas was released via a control valve.

The samples were mounted on a typical electron microscopy stub using a double-adhesive carbon tape. Each sample was coated with a thin layer of gold by a sputter gold coater (15~30 s deposition time, 15 mA current) (SC500 EMScope, Germany). Samples were observed with a field emission scanning electron microscope (Zeiss Supra 35VP FE-SEM, Germany) by with a voltage of 3 kV.

2.4.9 Histology and morphology of cell-seeded scaffolds

The migration of cultured cells within the scaffolds was determined using histology. Cell-seeded polymer discs were harvested, washed with PBS (×2) and fixed with 10% neutral buffered formalin (NBF) (Sigma Aldrich, UK) for 24 h at 4 °C. Following dehydration through a series of graded ethanol solutions, the samples were paraffin-embedded and horizontally cross-sectioned (2~4 μm), and the sections from the middle area (~ depth 250 μm from top) were analyzed with hematoxylin and eosin (H&E) staining. The stained samples were observed by stereo microscope imaging system (Nikon, SMZ 1500, Japan) fixed with a digital camera (INFINITY2-1C) at ×10, ×20, ×40 magnification. Average cell counting data was obtained on three 24×10\textsuperscript{4} μm\textsuperscript{2} field of view areas (×20, ×40 magnification) for each of the prepared slice samples. The stained samples were also observed by a digital slide scanner (Leica SCN400F, Germany) at ×40 magnification.

2.4.10 Gene expression via quantitative reverse transcriptase polymerase chain reaction (qPCR) analysis

Multipotent and differentiated cells in each scaffold were used for RNA collection, cells (n=4) were dissociated using TrypLE after removing the culture medium. The dissociated cells were incubated at 37 °C for 3 mins before being harvested into 15 ml tubes. Following this, cells were centrifuged at 800×g for 3 mins, the supernatant was removed completely, and the cell pellets were used for total RNA extraction from differentiated cells and knockdown cells using a Qiagen RNeasy mini-kit (Qiagen, UK). A high-capacity RNA-to-cDNA™ kit (Life Technologies, UK) was used for reverse transcription. For qPCR, all primers were designed using mRNA sequences taken from the NCBI nucleotide database. All primers were designed using the Roche online design tool. The sequences were copied into the Universal Probe Library for human application. Following this, candidate primers of 17~29 bps with annealing temperatures of 59~60°C and an amplicon of approximately 70~110 bps were generated automatically. The primer sequence was then copied into the NCBI Blast database to ensure that the primers matched the sequence of interest and shared no homology with any other genes. All primers were purchased from Invitrogen and were diluted with RNase/DNase-free water (Sigma, UK).

For qPCR, each individual reaction was mixed: 12.5 μl of BioMix Red (Bioline, UK), 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 1 μl of template, and 9.5 μl H\textsubscript{2}O. The template was cDNA and a
non-template control (i.e. H\textsubscript{2}O). Reactions were performed in an Eppendorf master cycler gradient (AppliedBiosystems, Germany) Real-Time PCR machine. The reaction conditions were as follows: initial denaturation (1 min, 95°C), 35 cycles [denaturation (30 s, 95°C); annealing (30 s, 60°C); elongation (45 s, 72°C)] and final elongation (5 min, 72°C).

2.5 \textit{In vivo} studies of cell culture on 3D scaffolds

2.5.1 \textit{In vivo} scaffold integration experiments

Following sterilization in 70% ethanol described above, three sets of PUU-POSS scaffolds with a 50% infilled density (40 mm× 40 mm× 2 mm, \(n=5\)) were subcutaneously implanted in adult male Sprague Dawley rats (Charles River Laboratories, United Kingdom), weighing 300~400 g at age of 6~8 weeks (\(n=30\)). All animals were kept in a temperature-controlled environment with a 12 h light/dark cycle and fed with a laboratory diet and tap water \textit{ad libitum}.

The study was conducted under a project license (70/7504) granted by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. All procedures were conducted in the comparative biology unit (CBU) of the Royal Free Hospital. The animals were preoperatively shaved, and ear marked accordingly, then anaesthetized with 4% isoflurane (induction) followed by 2% isoflurane (maintenance) by inhalation in combination with a 2:1 mixture of O\textsubscript{2}/N\textsubscript{2}O. A single incision large enough to allow insertion of the scaffolds was made and then closed with sub dermal interrupted sutures (Mersilk 3-0). The polymeric sheets were implanted slightly posterior to the scapulae to prevent any disruption to motor function and/or discomfort. All animals were monitored on a daily basis. At each time point, blood samples were taken by tail vein amputation for biochemical analysis, to indicate the liver (LFTs) and kidney function (U&Es).

The rats were then scanned with a laser Doppler perfusion imager and an oxygen sensor to examine blood flow through the implantation. Measurements of superficial scaffold vascularization were obtained using a LDI2-HIR laser Doppler scanner (Moor Instruments Ltd, Axminster, Devon, UK). No discomfort or attempts to excise the implants have been observed. At 4-, 8- and 12-weeks post-implantation, the rats were sacrificed by CO\textsubscript{2} asphyxiation.

2.5.2 Immunohistochemistry (IHC) and histology assessments

Fresh explant scaffolds (\(n = 3\) per group) were washed in Dulbecco's phosphate-buffered saline (DPBS) and fixed in 4% PFA in saline buffer overnight. Samples were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, UK) and blocked with 1% BSA (Sigma-Aldrich, UK) in DPBS. They were then incubated with rabbit-anti CD31 antibody (1/500 dilution; Abcam, Cambridge, UK) in 1% BSA in DPBS for 2 h at room temperature. A goat anti-rabbit Alexafluor-594 (1/500 dilution, Sigma-Aldrich, UK) in 1% BSA in DPBS was added for 1 h at room temperature. Adjacent sections were incubated with immunoglobulin G (IgG) antibody as negative control. Images were captured using a SPV8 confocal microscope (Leisca, Germany) at \(\times40\) magnification. Z-stacks were created with a 2 \(\mu\)m distance
between individual images. Z-stack image files were then read into IMARIS 7.6.3 analysis software (Bitplane Scientific, Switzerland) and were converted into three-dimensional representations.

For histological slide preparation, explants ($n = 3$ per group) were washed in Dulbecco’s phosphate-buffered saline (DPBS) and fixed in 10% neutral buffered formalin for 20 min. Briefly, explant samples were embedded in paraffin wax and cut into 4 μm thick sections using a rotary microtome, Leica RM2235 (Leica Microsystem Ltd., Milton Keynes, UK). They were then deparaffinised and stained with Hematoxylin and Eosin (H&E) and Masson’s trichome (M&T) capillary markers (i.e. CD31), macrophage markers CD86/CD68 (macrophage phenotype type I, M1), CD163 (macrophage phenotype type II, M2) and T-cell marker CD3/CD4, and evaluated using ImageJ (NIH, USA). Rat liver was used as positive control against CD68+, CD86+ and CD163+ staining, while rat spleen was used as positive control against CD3+ and CD4+ staining. Negative control was rat appendix. Histological sections were imaged using a digital slide scanner (Leica SCN400F, Germany) and evaluated using ImageJ (NIH, USA).

### 2.6 Conclusion

General materials and methods were elaborated in detail in this chapter. Synthesis and fabrication strategy of PUU-POSS scaffold material with involvement of newly-proposed 3D-TIPS method was discussed. Then, characterization of the scaffold properties from mechanical, thermal and material perspectives were conducted via a repertoire of techniques. Experimental procedures were also elaborated here regarding *in vitro* and *in vivo* studies of cell culture on scaffolds.
3.1 Introduction

Tissue-engineered scaffolds serve as a key component which should possess proper architecture and mechanical properties, apart from bioactivity, the ability to host seeded cells (i.e. adhesion, proliferation and differentiation), nonimmunogenicity, nontoxicity and noncarcinogenicity/nonteratogenicity. Various responsive polymeric materials have been developed for tissue engineering as model to study stiffness effects in cell cultures. Unfortunately, they are still constrained by the coupling effect of molecular structural change and elasticity, which often has a narrow range of tuneable stiffness. Block-copolymers, such as polyurethane (PU) elastomers, can demonstrate a wide range of tuneable physical and mechanical properties without changes to their underlying molecular structure simply by varying the method of self-assembly (i.e. physical phase transition, phase-separation) [189], apart from chemical modification of chain block ratios [190]. Their elasticity and biocompatibility [92] allow for the creation of a diverse family of high-performance polymers composed of various soft and hard segments with unique nano- and microstructures, many of which have been employed in various medical devices and soft implants. In particular, solution-based poly(urea-urethane)-polyhedral silsequioxane (PUU-POSS) has long demonstrated outstanding compliance, fatigue resistance, biocompatibility and biostability in long-term implantable cardiovascular devices due to its stronger hydrogen chain bonding via urea groups [191, 192]. PUU-POSS is an elastomeric hybrid nanocomposite with a unique nanostructure that offers excellent hyperelasticity and makes it suitable for soft implants and scaffolds [94].

Thermally induced phase-separation (TIPS), as a conventional method has been widely used, performs well in controlling the pore size, pore morphology, and pore interconnectivity of scaffolds by varying the processing conditions [149, 150]. But it pales when dealing with larger complex constructs. The advent of 3D printing has presented the biomedical community with an opportunity to design and manufacture complex 3D organ-like shapes with well-defined macroscopic porous architectures based on patient-
specific dimensions (e.g. from X-Ray, computerised tomographic (CT) and magnetic resonance imaging (MRI) scans), without the need for conventional tooling or moulding [193, 194]. Despite this promise, the main caveat for biomedical applications lies in the limitation on the types of biologically active soft biomaterials available for 3D printing. Due to the high boiling temperatures of dimethylacetamide (DMAC) used as solvent and the slow coagulation process in water, PUU-POSS cannot be printed and does not easily lend itself to the creation of large porous constructs with complex shapes [94, 192].

To overcome these limitations of conventional TIPS and 3D fused modelling deposition (FDM) printing, such as non-uniform porous structure, low resolution of pores and limited printable materials, it is detailed herein the manufacture of thermoresponsive non-degradable PUU-POSS nanohybrid scaffolds with stiffness softening using indirectly 3D printing approach combined with thermal induced phase-separation (3D-TIPS) technique. Well-defined micro-channels can be digitally programmed to guide the phase-separation of solution-based PUU-POSS, leading to the creation of full-scale scaffolds, with hierarchically interconnected pores and controlled porosity, crystallinity and mechanical properties. It is observed stiffness softening to an intrinsic soft rubbery phase at body temperature with maintenance of the preformed complex shape, through reverse self-assembling crossing over a wide range of chain relaxation times. The biological in vitro interactions during dynamic mechanical relaxation of these scaffolds demonstrate their promise as personalized and biologically responsive soft scaffolds and implants.

3.2 Materials and methods

3.2.1 3D thermal induced phase-separation (3D-TIPS) manufacturing polyurethane urea scaffolds

Ethylenediamine and polyhedral oligomeric silsesquioxane (POSS) co-terminated poly(urea-urethane) (PUU) polymer (PUU-POSS) in N, N-dimethylacetamide (DMAC) solution was synthesised as needed, using methods described previously in Section 2.2.1 Chapter 2. The molecular structure of PUU-POSS can be seen in Figure 1.2 Chapter 1.

Design and fabrication of 3D printing Poly (vinyl alcohol) (PVA) preform were detailed in Section 2.2.2 Chapter 2. PVA preforms were designed in OpenSCAD (v. 2015.03), exported as .stl files and sliced into consecutive 200 µm layers with Slic3r (v. 9.9) for 3D printing with a 50% infill orthogonal density of resolution 400 µm × 400 µm. A PVA filament of 1.75 mm in diameter was extruded with a fusion deposition printer (Active X1; Active 3D Printers Ltd., UK) at 210 ºC at 150 mm.s⁻¹ for X/Y printing speed and at 25 mm.s⁻¹ for Z printing speed. The nanohybrid polymeric solution was then injected through a surface punctured hole into the 3D printed PVA preforms, used as water soluble negative moulds.
Three groups of scaffolds were manufactured at different coagulation temperatures and post thermal treatment as described in Table 3.1. The first group of filled preforms (Cryo Coagulation, CC) were kept overnight at −20°C and then immersed into deionised ice water for 24 hours, with regular water changes at room temperature and subjected to magnetic stirring until the PVA had been dissolved. The second group (Cryo Coagulation + Heating, CC+H) followed the same process of CC group but had additional heat treatment in deionised water at 40°C for 3 hours. The final group (Room Temperature Coagulation + Heating, RTC+H) were immersed into deionised water at room temperature (25°C) and soaked for 24 hours, with regular water changes and magnetic stirring, following by heat treatment at 40°C for 24 hours until the PVA dissolved. All scaffolds were stored in sterile de-ionised water until they were required.

The strut thickness of PUU-POSS scaffolds varied depending on the infill density and printing resolution of PVA preform. In the case of 50% infill density and 200 µm slicing thickness, the average thickness of the strut in-plane (X- and Y-axis) is between 146-149 µm and Z-axis is between 118-127 µm, with various tolerances depending on the processing conditions as listed in Table 3.2.

Reconstructed digital. stl file images of an ear and nose were obtained from CT-scan images, sliced with a 50% infill density, and printed as PVA preform. PUU-POSS solution was injected into each preform, and the preform was washed as outlined for the RTC+H groups above, to produce anatomically shaped polymer scaffolds.

**Table 3.1 Process conditions of 3D-TIPS**

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>PUU-POSS solution filled PVA preform</th>
<th>Coagulation conditions</th>
<th>Thermal treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryo-Coagulation, CC</td>
<td>−20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
<td>No thermal treatment</td>
</tr>
<tr>
<td>Cryo-Coagulation + Heating, CC+H</td>
<td>−20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
<td>40°C water for 3 h</td>
</tr>
<tr>
<td>Room Temperature Coagulation + Heating, RTC+H</td>
<td>Room temperature, 25°C</td>
<td>Room temperature, 25°C water for 24 h</td>
<td>40°C water for 24 h</td>
</tr>
</tbody>
</table>
Table 3.2 Dimensions of 3D printed PVA preform and PUU-POSS scaffolds made by 3D-TIPS

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>X-Strut thickness (n=10)</th>
<th>Y-Strut thickness (n=10)</th>
<th>Z-Strut thickness (n=10)</th>
<th>Sample Size, (L×W×T, mm) (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% infill PVA preform (designed mould)</td>
<td>400</td>
<td>400</td>
<td>200</td>
<td>60×12×4</td>
</tr>
<tr>
<td>50CC</td>
<td>159±16</td>
<td>157±9</td>
<td>118±19</td>
<td>61×13×4</td>
</tr>
<tr>
<td>50CC+H</td>
<td>143±12</td>
<td>150±8</td>
<td>121±14</td>
<td>60×11×4</td>
</tr>
<tr>
<td>50RTC+H</td>
<td>137±7</td>
<td>140±11</td>
<td>127±10</td>
<td>59×13×4</td>
</tr>
</tbody>
</table>

3.2.2 Static and dynamic mechanical studies

These studies were described previously as referred in Section 2.3.1 Chapter 2.

3.2.3 Characterisation of scaffold structure

General descriptions were previously referred in Section 2.3.2 Chapter 2. The phase transitions of the scaffolds were measured from −70°C to 230°C using differential scanning calorimeter (DSC Q1000, TA Instrument, Germany) at a scan rate of 10°C/min. The phase structure was further examined by wide-angle X-Ray diffraction (WAXD, Bruker D8 Advance, Germany). The top surfaces and cross sections of dried scaffolds had their surface and cross-sectional features examined at room temperature using an optical microscope (Olympus DSX500, UK), and under a field emission scanning electron microscope (FE-SEM, Zeiss Supra 35VP, Germany). The nanophase structure of PUU-POSS was observed using high resolution TEM (JEOL2100 field emission gun transmission electron microscope, FEG-TEM, Japan). The porosity of the membranes was determined using gravimetry. The dimensions of the scaffold discs (16 mm in diameter and 3 mm in thickness) were cut using a cutter. The weight of the sample was measured using a high sensitivity balance (resolution 0.01mg). The apparent density and porosity of the scaffolds were calculated using the equation E3.1. A mercury intrusion porosimeter, (PoreMaster 60GT, Quantachrome, UK) was used to characterise the pore structure including the pore size, pore volume, size distribution, surface area of the freeze-dried scaffolds.
The porosity of PUU-POSS scaffolds was calculated using the equation below,

\[ \rho = (1 - \frac{d_a}{d_b}) \times 100\% \]  

(E3.1)

where the bulk density, \( d_b \), of PUU-POSS was taken as 1.15 g/cm\(^3\) (1150 kg/m\(^3\)), the apparent density, \( d_a \) (i.e., scaffold density), was calculated using the weight, \( W_a \) (g) and volume, \( V_a \) (cm\(^3\)) of each sample, \( d_a = \frac{W_a}{V_a} \). Six discs (of diameter 1.6 cm) were cut from each scaffold using a pre-shaped cutter. Average radius and height were measured for each disc to the nearest 0.01 mm using a digital caliper.

**Note:** The \( d_b \) of PUU-POSS, 1150 kg/m\(^3\), was measured from the bulk polymer membrane in amorphous rubber phase made by casting of PUU-POSS solution through solvent evaporation. Therefore, it is accurate to apply this bulk density for calculating the porosity of the RTC+H and CC+H scaffolds (with little residual of crystalline structure left after thermal treatment). However, it is only approximate to apply 1150 kg/m\(^3\) of PUU-POSS in amorphous rubber phase to estimate the porosity of semicrystalline CC scaffold, because it is unlikely to obtain a solid film of PUU-POSS with the same semicrystalline phase or 100% crystallinity based on currently available manufacturing conditions. In addition, the crystallinity of CC group was measure about 37.61 % by wide-angle X-Ray diffraction (WAXD), which may have limited contribution to the change of its density.

### 3.2.4 Cell culture, cell proliferation and immunochemical staining

The protocol of primary human dermal fibroblast (HDF) cell expansion, sterilization of the scaffolds and optimization of cell seeding density on the scaffolds are presented in Section 2.4 Chapter 2. The metabolic activity of cells was monitored to determine cell viability through alamarBlue® (AB) (Serotec Ltd, Kidlington, UK) according to manufacturer's instructions. One day post seeding, scaffolds were transferred to a new 48-well plate to avoid false positive readings from cells adhering to the tissue culture plastic rather than the scaffold. Readings were taken at day 1, 3, 7, 10 and 14. The alamarBlue™ reading in cell culture medium of TCP/polymer scaffold without cell seeded was used as a negative control. The total deoxyribonucleic acid (DNA) content quantification was determined using the fluorescent Hoechst 33258 stain and the amount of extracellular acid-soluble collagen (types I - V) was measured using the Sircol™ assay (Biocolor, UK) at each time point as day 1, 3, 7, 10 and 14.

Fibroblast cytoskeleton and attachment to the various PUU-POSS scaffolds was studied as shown in Section 2.47 Chapter 2

### 3.2.5 Data analysis

Statistical analysis of the results was performed using Graph-Pad Prism 6 (GraphPad Software San Diego, CA). Statistical significance was calculated by 2-way ANOVAs (for comparisons across more
than two groups) analysis of variance (ANOVA), with Tukey multiple comparison post-hoc analysis where a value of $p < 0.05$ was considered statistically significant.

### 3.3 Results

#### 3.3.1 Fabrication of hierarchical interconnected porous PUU-POSS scaffolds

The 3D-TIPS technique comprises two low-cost manufacture stages to produce a final soft PUU elastomer construct with its complex shape and hierarchical interconnected porous structure (Figure 3.1). Solution-based poly(urea-urethane) (PUU) nanohybrid terminated by polyhedral oligomeric silsesquioxane (POSS) was synthesised in Section 2.2.1 Chapter 2. Preforms were printed from water-soluble polyvinyl alcohol (PVA) by using an inexpensive fused deposition modelling (FDM) extruder. Printed preforms were injected with PUU-POSS and submerged into deionised water, resulting in confined coagulation and uniform phase separation of PUU-POSS prior to PVA dissolution, driven by diffusion water and solvent along the interconnected micro-channels (Figure 3.1A). The resulting scaffold was inversely shaped internally to the PVA preform with continuous interconnection, overcoming the weak interfaces created when polymer layers are printed on each other directly. Anonymised CT scans of facial structures (nose and ear) were used to produce bespoke, patient-specific preforms and their daughter scaffolds (Figure 3.1B) and evaluated by optical, micro-CT and scanning electron microscope (SEM) (Figure 3.1C-3.1H).

PUU-POSS scaffolds demonstrated controllable and hierarchical porous structures (Figure 3.1 C-3.1H), with regularly ordered and completely interconnected pores of 300-400 µm in-plane and 100-400 µm cross-section, accommodating the inverse porous structure of the infill density and resolution of the PVA preform. A random finer porous surface structure was evident throughout the scaffold strut at a few microns to nanometre scale (Figure 3.1G & 3.1H) resulting from uniform phase separation of PUU-POSS solution within the preform multi-micron-channels, which are significant advantages over FDM printing techniques and TIPS process alone. Macro- to microscopic modifications to scaffold porosity were easily achieved by varying coagulation condition and the infill density of the PUU-POSS scaffolds (Figure 3.2 and Table 3.3 and Table 3.4). Figure 3.2 A and 3.2 B show hierarchical interconnected porous structures of 3D-TIPS scaffolds with digitally defined macro-pores to micro- to nano-pores generated during phase separation of the polymer solution. SEM images of cross section of 50CC in Figure 3.2 B demonstrate a bead-like network of pores with diameters ranging from a few microns to nanometers generated during the cryo-process. It also showed an irregular surface with a minimal number of open nano-pores (Figure 3.2 B) due to faster diffusion and phase separation at room temperature and shrinkage during post thermal treatment, resulting in the formation of a dense surface with fewer pores.
Figure 3.1 Schematic outlining the multi-micro-channel-controlled phase separation of 3D PUU-POSS scaffolds with hierarchical interconnected porous structure. (A) An overall schematic showing how the 3D printed preform phase separation (3D-TIPS) technique works in relation to patient care, from CT scan to final polymer mesh implant, in order to create uniquely shaped polymer meshes to suit individual patient needs. (B) A schematic demonstration of the principle of multi-micro-channel-controlled 3D phase separation in the 3D-TIPS technique stepwise from left to right, with the preform shown in blue and the nanocomposite polymer shown in yellow. (C) Photo, (D) Optical microscopic, (E) Micro-CT, (F-G) SEM images of the surface and (H) the cross section.
Table 3.3 Physical and mechanical properties of PUU-POSS scaffolds with various infill densities.

<table>
<thead>
<tr>
<th>Scaff old</th>
<th>Infill Dens ity, %</th>
<th>Scaff old Dens ity, dₖg/m³</th>
<th>Total Porosity, 100%</th>
<th>Tensile modulus, MPa, at 50% strain</th>
<th>Ultimate Strength, MPa</th>
<th>Tensile strain at break, %</th>
<th>Toughness, J/m²·m³×10⁴</th>
<th>Compre ssion Strength, MPa</th>
<th>Compression Modulus, MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>80</td>
<td>44 ± 3</td>
<td>96.2 ± 0.3</td>
<td>4.39 ± 0.63</td>
<td>0.80 ± 0.06</td>
<td>58 ± 11</td>
<td>32 ± 6</td>
<td>0.54 ± 0.02</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>CC</td>
<td>70</td>
<td>40 ± 3</td>
<td>96.5 ± 0.3</td>
<td>2.48 ± 0.20</td>
<td>2.26 ± 0.23</td>
<td>124 ± 10</td>
<td>165 ± 15</td>
<td>0.48 ± 0.01</td>
<td>0.75 ± 0.01</td>
</tr>
<tr>
<td>CC</td>
<td>60</td>
<td>37 ± 5</td>
<td>96.6 ± 0.7</td>
<td>1.57 ± 0.13</td>
<td>2.10 ± 0.10</td>
<td>177 ± 9</td>
<td>211 ± 59</td>
<td>0.34 ± 0.01</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>CC</td>
<td>50</td>
<td>34 ± 4</td>
<td>96.8 ± 0.4</td>
<td>0.98 ± 0.14</td>
<td>1.33 ± 0.09</td>
<td>179 ± 8</td>
<td>137 ± 23</td>
<td>0.33 ± 0.03</td>
<td>0.48 ± 0.08</td>
</tr>
<tr>
<td>CC</td>
<td>40</td>
<td>30 ± 6</td>
<td>97.4 ± 0.5</td>
<td>0.78 ± 0.06</td>
<td>1.06 ± 0.05</td>
<td>162 ± 6</td>
<td>118 ± 19</td>
<td>0.17 ± 0.04</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>CC</td>
<td>30</td>
<td>27 ± 3</td>
<td>97.7 ± 0.3</td>
<td>0.50 ± 0.06</td>
<td>0.63 ± 0.03</td>
<td>146 ± 5</td>
<td>67 ± 16</td>
<td>0.10 ± 0.02</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>CC+H</td>
<td>80</td>
<td>56 ± 8</td>
<td>95.1 ± 0.7</td>
<td>1.04 ± 0.09</td>
<td>1.11 ± 0.12</td>
<td>119 ± 14</td>
<td>53 ± 10</td>
<td>0.38 ± 0.01</td>
<td>0.56 ± 0.01</td>
</tr>
<tr>
<td>CC+H</td>
<td>70</td>
<td>51 ± 4</td>
<td>95.5 ± 0.3</td>
<td>0.74 ± 0.04</td>
<td>0.89 ± 0.02</td>
<td>199 ± 16</td>
<td>76 ± 18</td>
<td>0.34 ± 0.04</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>CC+H</td>
<td>60</td>
<td>49 ± 3</td>
<td>95.8 ± 0.3</td>
<td>0.53 ± 0.05</td>
<td>0.74 ± 0.05</td>
<td>196 ± 4</td>
<td>89 ± 20</td>
<td>0.26 ± 0.02</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>CC+H</td>
<td>50</td>
<td>45 ± 5</td>
<td>96.1 ± 0.5</td>
<td>0.43 ± 0.02</td>
<td>0.77 ± 0.05</td>
<td>236 ± 19</td>
<td>113 ± 27</td>
<td>0.21 ± 0.01</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>CC+H</td>
<td>40</td>
<td>41 ± 4</td>
<td>96.5 ± 0.3</td>
<td>0.48 ± 0.04</td>
<td>0.47 ± 0.03</td>
<td>195 ± 7</td>
<td>84 ± 13</td>
<td>0.11 ± 0.01</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>CC+H</td>
<td>30</td>
<td>37 ± 2</td>
<td>96.8 ± 0.2</td>
<td>0.38 ± 0.05</td>
<td>0.42 ± 0.05</td>
<td>170 ± 5</td>
<td>59 ± 16</td>
<td>0.13 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>RTC+ H</td>
<td>80</td>
<td>48 ± 10</td>
<td>95.8 ± 0.8</td>
<td>0.69 ± 0.03</td>
<td>1.07 ± 0.06</td>
<td>229 ± 5</td>
<td>229 ± 21</td>
<td>0.35 ± 0.01</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>RTC+ H</td>
<td>70</td>
<td>43 ± 4</td>
<td>96.2 ± 0.4</td>
<td>0.56 ± 0.06</td>
<td>1.08 ± 0.02</td>
<td>264 ± 16</td>
<td>264 ± 47</td>
<td>0.25 ± 0.02</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>RTC+ H</td>
<td>60</td>
<td>39 ± 5</td>
<td>96.6 ± 0.4</td>
<td>0.51 ± 0.07</td>
<td>1.01 ± 0.07</td>
<td>285 ± 15</td>
<td>285 ± 33</td>
<td>0.22 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>RTC+ H</td>
<td>50</td>
<td>38 ± 3</td>
<td>96.7 ± 0.3</td>
<td>0.44 ± 0.06</td>
<td>0.67 ± 0.03</td>
<td>146 ± 15</td>
<td>146 ± 12</td>
<td>0.17 ± 0.02</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>RTC+ H</td>
<td>40</td>
<td>33 ± 5</td>
<td>97.1 ± 0.4</td>
<td>0.48 ± 0.07</td>
<td>0.55 ± 0.04</td>
<td>118 ± 22</td>
<td>118 ± 37</td>
<td>0.12 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>RTC+ H</td>
<td>30</td>
<td>29 ± 3</td>
<td>97.5 ± 0.3</td>
<td>0.29 ± 0.07</td>
<td>0.37 ± 0.02</td>
<td>66 ± 18</td>
<td>67 ± 9</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>
Figure 3.2 Morphology structure of 3D PUU-POSS scaffolds with varying infill density and processing temperatures under different processing conditions. (A) Optical images of surface and cross-section of the scaffolds with infill densities 80-30% made by 3D-TIPS. (B) SEM images of cross-sections of CC, CC+H and RTC+H of PUU-POSS scaffolds with 50% infill density. (C) Schematic of the structured 3D PUU-POSS scaffolds, alongside the pore unit cell dimensions.
Table 3.4 Pore size and size distribution by a mercury intrusion porosimeter (n=1)

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Pore Diameter, nm</th>
<th>Pore Volume, cm³/g</th>
<th>Relative Pore Volume, %</th>
<th>Surface Area, m²/g</th>
<th>Relative Surface Area, %</th>
<th>Porosity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>50CC</td>
<td>500,000 to 1000</td>
<td>29.8</td>
<td>58.5</td>
<td>1.5</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 to 100</td>
<td>11.3</td>
<td>22.1</td>
<td>48.7</td>
<td>83.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 to 3</td>
<td>9.9</td>
<td>19.4</td>
<td>8.3</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>50.9</td>
<td>100</td>
<td>58.5</td>
<td>100</td>
<td>98.3</td>
</tr>
<tr>
<td>50CC+H</td>
<td>500,000 to 1000</td>
<td>31.3</td>
<td>75.8</td>
<td>1.5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 to 100</td>
<td>6.6</td>
<td>15.9</td>
<td>18.8</td>
<td>76.8</td>
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<tr>
<td></td>
<td>100 to 3</td>
<td>3.5</td>
<td>8.4</td>
<td>4.2</td>
<td>17.2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>41.3</td>
<td>100.0</td>
<td>24.5</td>
<td>100.0</td>
<td>97.9</td>
</tr>
<tr>
<td>50RTC+H</td>
<td>500,000 to 1000</td>
<td>48.6</td>
<td>95.2</td>
<td>2.7</td>
<td>58.5</td>
<td></td>
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<tr>
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<td></td>
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<tr>
<td></td>
<td>100 to 3</td>
<td>2.5</td>
<td>4.8</td>
<td>1.9</td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>51.1</td>
<td>100.0</td>
<td>4.6</td>
<td>100.0</td>
<td>98.3</td>
</tr>
</tbody>
</table>

3.3.2 Tuneable hierarchical structure and hyperelasticity of the 3D-TIPS scaffolds

Using different phase separation temperatures, it was controlled PUU-POSS scaffold hierarchical porosity and phase-structure to produce scaffolds with a wide range of mechanical properties whilst maintaining a constant external architecture. The three groups of 50% infill density PUU-POSS scaffolds using differing thermal conditions were manufactured (in Table 3.1-3.4). PUU-POSS coagulation and micro-phase separation were varied by controlling water and solvent diffusion rates within the micro-channels of a PVA preform and dissolution rate of PVA at different temperatures. The 50RTC+H scaffolds underwent fast coagulation (liquid-liquid phase separation) at room temperature (25°C) following thermal annealing in water at 40°C; the 50CC scaffolds underwent slow cryo-coagulation (liquid-solid phase separation) of frozen PUU-POSS solution (at −20°C overnight) in ice water at 0°C, and the 50CC+H scaffolds underwent the same process to 50CC followed by thermal annealing in water at 40°C for 3 hours.
Figure 3.3 Phase transitions, crystalline structure, pore size distribution, morphology and nanophase structure of 3D PUU-POSS scaffolds (50% infill density) with varying processing temperatures. (A) Differential scanning calorimetry (DSC) traces of scaffold meshes. (B) Wide-angle X-ray diffraction (WAXD) analysis of scaffold meshes. (C) Mercury porosimeter measurement of pore size and distribution. (D1-F2) SEM, and (D3-F3) HRTEM images demonstrating variations in micro-/nano-phase separation of PUU-POSS scaffolds with differing phase separation processes (the inset presents fast Fourier transform (FFT) pattern). (D1, D2, D3) 50CC, (E1, E2, E3) 50CC+H, (F1, F2, F3) 50RTC+H.

50RTC+H scaffolds demonstrated the glass transition temperature ($T_g$) of -30 to -35°C on DSC (Figure 3.3 A), suggesting a soft amorphous rubber-phase structure throughout. WAXD analysis showed three background halo peaks centred at approximately $2\theta$=19.9°, 31.3° and 41.3°, corresponding to a nanophase structure driven by different hydrogen bonds between hard-hard and hard-soft segment interactions at varying distances [195] (Figure 3.3 B). HRTEM 50RTC+H group image (Figure 3.3 F3) also supported a quasi-random nanophase structure of soft and hard segments, with evidence of a
diffusion halo from electron diffraction. It is noted that the fine, dark and dense hard nano-domains, resulting from selectively RuO4 stained urethane regions, self-assembled and interconnected through bright and continuous soft polycarbonate domains. No crystals and clusters of POSS nanocage were observed, indicating well distributed as PUU chain ends.

50CC scaffolds demonstrated a sharp melting peak appeared at 45~50°C on DSC, with the same T_g at -30 °C attributed to the amorphous region of the soft segment chains, as well as a smaller broad peak at 190°C likely corresponding to the second T_g of the hard segment chains (Figure 3.3 A). On XRD analysis, two sharp Bragg diffraction peaks at 2θ=20.0° and 23.4° were superimposed onto a broad amorphous halo at 2θ=19.9° with inter-planar spacing (d-spacing) of 0.44 nm and 0.38 nm, suggesting the lateral distances in the interfaces of crystallized soft segments (Figure 3.3 B). HRTEM 50CC group image (Figure 3.3 D3) further supported a structure of bright polycarbonate crystalline domains with distinctly ordered lattice structures within the random nanophase structure, with an electron diffraction pattern with d-spacing 0.43 nm and 0.37 nm in agreement with XRD analysis.

In 50CC+H scaffolds, the sharp melting peaks seen in 50CC scaffolds on DSC almost disappeared after thermal treatment, with the reappearance of the clear step of T_g at -35°C suggesting reversion back to a rubber phase with a small residual peak at the melting temperature likely due to unrelaxed crystal regions from the soft segment chains (Figure 3.3 A). On XRD analysis, the crystal structure also almost disappeared (Figure 3.3 B), through relaxing the long-distance order to a quasi-random amorphous structure with a similar diffraction profile to 50RTC+H comprising three broad halo peaks. HRTEM 50CC+H group (Figure 3.3 E3) confirmed a quasi-random nanophase structure after the thermal relaxation at 40°C, and it further confirmed that the crystalline peaks seen in 50CC scaffolds are from crystalline soft segment domains.

The scaffolds are lightweight with porosity between 95~98% and density between 27-56 kg/m³ depending on the infused density and processing temperature (Table 3.3). Obviously, the scaffold density decreased, and the porosity increased with increasing the infill density. With the same infused density, CC group has the lowest density and highest porosity, and CC+H group in opposite. Further measurement of the pore size and distribution of the scaffold with 50% infill density by mercury porosimetry revealed the insight difference (Figure 3.3 C, Table 3.4). 50RTC+H scaffolds had a wide distribution of pore sizes, with 95.2% relative pore volume provided by one predominate broad peak with pore diameter from 400 to 10 μm and the rest 4.8% compromising of small pore diameters ranging from 10 μm to 3 nm (Figure 3.3 C, Table 3.4), whilst SEM images showed an irregular surface with a minimal number of open nano-pores (Figure 3.3 F1-F2). This may be due to faster diffusion and phase separation occurring at room temperature and shrinkage during post thermal treatment, resulting in the formation of a skin with fewer pores. Contrastingly, the 50CC scaffolds showed greater hierarchy in its porous structure, with distinct three peaks ranging from 400 to 10 μm, 10 μm to 100 nm and 100 to 3 nm, sharing 58.5, 22.1 and 19.4% relative pore volume and contributing to 2.7, 83.2 and 14.2% surface area (Figure 3.3 C, Table 3.4). SEM images demonstrated a bead-like network of pores with diameters ranging from a few microns to nanometres stacked on top of each other (Figure 3.3 D1-D2), which is
likely due to spherulite formation during the cryo-process. 50CC+H scaffolds had a similar bead-stacking morphology and pore size distribution, with fewer pores at 10 µm ~ 3 nm, owing to shrinkage during post thermal treatment (Figure 3.3 E1-E2). It is noticed that the porosity of scaffolds of 50% infill density (only one test) calculated from the pore volume in Table 3.4 is slightly higher than that measured by gravimetry in Table 3.3, which may be attributed to different accuracy of test methods. The effects of soft elastomer and mismatch of pore structure model in mercury porosimetric measurement and analysis should be taken into account.

With tuneable phase and porous structures, the three scaffold groups with various printed infill densities of the PUU-POSS scaffold possessed distinct mechanical properties, supporting a capability of 3D-TIPS technique to manufacture a range of tissue-engineering scaffolds (Figure 3.4 and Table 3.3). Figure 3.4 shows tensile and compression mechanical properties of the scaffolds with different infill densities and processing conditions. Despite of highly porous structure with the porosity between 95-98%, the scaffolds showed ductile mechanical behaviour with high fracture strain from over 50% to 300% under tensile stress (Figure 3.4 A). Tensile modulus (E) was the highest for each group in the 80% infill density (Figure 3.4 B), measuring 4.4 ± 0.6 MPa in the CC, 1.0 ± 0.1 MPa in the CC+H and 0.7 ± 0.1 MPa in the RTC+H groups, with a stepwise decrease in modulus with increasing infill density across all three groups. The modulus in the CC group was significantly higher than the lowest in RTC+H group, for each infill density. The ordered crystalline structure in the CC group contributed to high modulus (×15 times higher than the lowest in 30% RTC+H), and these properties lost in the CC+H group once crystalline structure was relaxed through thermal treatment. However, the other tensile mechanical properties of the 80% CC infill density scaffold including ultimate strength, strain and toughness appeared underperformance, which may be attributed from less entanglements of chain during the freezing process and the poor uniformity of microporous structure generated during coagulation in the presence of less water channels.
Figure 3.4 Mechanical properties of 3D PUU-POSS scaffolds with varying infill density and processing temperatures. (A) Typical tensile stress-strain profiles of three groups of the scaffolds, CC, CC+H and RTC+H with varying infill density. (B) Tensile modulus. (C) Ultimate tensile strength. (D) Tensile strain at break. (E) Tensile toughness of the scaffolds. (F) Compression modulus. (G) Compression strength of the scaffolds with increasing infill density.

Ultimate tensile strength (UTS) for scaffolds (Figure 3.4 C) was the highest for the CC group in the 70% infill density preforms at 2.3 ± 0.2 MPa. For each group, there was a stepwise decline following peak UTS with increasing infill density except for 80% CC due to an increase of the non-uniformity of the scaffolds at low infill density. At each infill density, UTS for the CC group was significantly higher than
that for the CC+H and RTC+H groups. Strain at break peaked at different infill density for each group, with the highest in the 60% RTC+H group at 285 ± 15% and the 50% infill densities of the CC and CC+H groups, at 178 ± 8% and 236 ± 19% respectively. It is also noticed that the strain at break of the RTC+H group at low infill density (up to 60%) was significantly higher (Figure 3.4 D) compared to that in CC and CC+H groups, owning to the formation of dense skin in RTC+H group from fast phase separation at the interface at the RT. However thermal treatment also substantially improved the ultimate strain of the CC group, outperforming the other groups at the high infill density which may be contributed by dense packing between the chains after heating, in evidence of a shrinkage of 50CC+H group with a negative volume swelling ratio, −18.0%, compared to 50CC, −0.9%, Table 3.5. Tensile toughness (Figure 3.4 E) demonstrated some similar trend, with the RTC+H group exhibiting higher toughness than the CC or CC+H groups due to its lower porosity and dense skin. Toughness was the highest in the 40% RTC+H scaffold at 285 ± 33 Jm⁻³10⁴, higher than the maximum in the 60% CC group, at 211 ± 59 Jm⁻³10⁴ and 50% CC+H at 113 ± 27 Jm⁻³10⁴. This superior hyper elasticity in the scaffolds was due to the continuous soft rubbery phase reinforced by a unique hard segment nanostructure. As expected, variations in the infill density dramatically affected the compression mechanical properties of the scaffolds, with greater compression modulus and strength for high infill densities due to less digitally defined macro porous structure (Figure 3.4 F-G, and Tables 3.3). Cryo-Coagulation also substantially increased the compression modulus and strength due to the formation of semi-crystalline ordered structures within the CC scaffolds, compared to CC+H and RTC+H with thermal treatment (Figure 3.4 F-G).

3.3.3 Thermoresponsive stiffness softening at body temperature

The scaffold underwent stiffness relaxation with a limited volume change at a swelling ratio varying from −3.3 vol% (50CC+H) to 20.6 vol% (50CC) (Table 3.4) when incubated in standard cell culture conditions for 28 days. The level of the stiffness changes depended on the initial 3D-TIPS (Figure 3.5 and Table 3.6) in the correlation between the structure and mechanical properties by comparing the scaffolds with 50% infilled density as produced (day 0) and after incubation for 28 days at 37°C. The significant reductions of tensile modulus (46%) and strength (57%) of 50CC group with initial high stiffness on day 28 demonstrate pronounce viscoelastic behavior, resulting in stiffness softening in response to the incubation body temperature (Figure 3.5 A-C). Rigid 50CC scaffolds gradually softened at 37.5 °C and stabilised over 3 weeks with around 50% reduction in both tensile modulus and ultimate tensile strength (Table 3.8), whilst 50CC+H showed a slight relaxation in tensile modulus with few changes in other mechanical properties and soft 50RTC+H scaffolds remained stable with no obvious changes (Figure 3.5 D-E). Despite this, scaffold toughness was not significantly altered due to compensation from an increase in fracture strain (Figure 3.5 F-G). In the compression test, the similar isothermal stiffness softening of 50% infill CC (50CC) scaffolds was observed at cell culture condition (i.e. 37.5°C). Figure 3.5 H-I shows that both the compression modulus and strength of the 50CC and 50CC+H scaffolds gradually reduced, with 54% and 48% reduction in the compression modulus, and 38% and 18.2% reduction in the compression strength for 50CC and 50CC+H respectively in the first 14 days of incubation at 37.5 °C, and then reached a similar level to that of the 50RTC+H group by 28 days (p-
value non-significant) (Table 3.8).

Figure 3.5 Stiffness softening of PUU-POSS scaffolds with a 50% infill density with different 3D-TIPS processing conditions after incubation at 37.5 °C for up to 28 days (n=6) by static mechanical tensile and compression tests. (A-C) Typical tensile stress-strain curves of three groups of the scaffolds, CC, CC+H and RTC+H at day 0 and day 28. (D) Tensile modulus. (E) Ultimate tensile strength. (F) Facture strain. (G) Tensile toughness. (H) Compression modulus. (I) Compression strength.
This stiffness relaxation was also evident on dynamic tensile mechanical testing in over $1 \times 10^6$ cycles (Figure 3.6 A-C, and Table 3.6). 50CC scaffolds demonstrated slow stress damping and reduction of hysteresis loop areas with increasing cyclic number at constant 25% strain in response to the transition from semi-crystalline to quasi-random nanostructured rubbery structures at body temperature. In contrast, the soft rubbery 50RTC+H scaffolds displayed linear hyperelasticity under much lower cyclic stresses with almost full recovery of their original forms on load removal. 50CC+H scaffolds also showed recoverable nearly-linear stress-strain characteristics, with only a trace of hysteresis loop indicating residual inelastic energy after thermal treatment. After one million cycles, three types of scaffolds all reached to similar reversible linear hyperelasticity.

The stiffness softening and hyperelasticity of the scaffolds was also clearly magnified by reduction of the cyclic loading (i.e., 200 cycles) before and after isothermal relaxation at 37.5°C for 28 days (Figure 3.6 D-F). All samples displayed reduced hysteresis values and gradually established by reaching their intrinsic elasticity after compression cyclic loading or isothermal annealing (Table 3.6). 50CC scaffolds demonstrated the largest area of hysteresis loop as produced, but gradually became softer with increasing reversible compliance through reduction of the loss energy under lower compression stress after 28-day incubation. In contrast, the 50RTC+H scaffolds did not change significantly after 28-day incubation, showing almost similar reversible hyperelasticity as produced under much lower cyclic load. 50CC+H scaffolds showed a smaller scale of stiffness softening, with only a trace of hysteresis loop indicating residual inelastic energy after isothermal annealing.

The dynamic response of the scaffolds was further evaluated on dynamic compression mechanical testing at increasing cycles (Figure 3.6 G-I). Typical hysteresis of stress and strain loops and changes in their configuration appeared when 50CC scaffolds were subjected to higher cyclic stresses for over $2 \times 10^6$ times at 37°C (Figure 3.6 G). A slow stress damping and reduction of hysteresis loop area was measured at increasing number of cycles in response to the transitions from 3D order semi-crystalline to quasi-random nanophase structures observed in the 50CC sample. In contrast, soft rubbery 50RTC+H scaffolds displayed highly reversible entropic hyperelasticity under low cyclic load, about x 6 lower, compared to 50CC and 50CC+H scaffolds on day 0 (Figure 3.6 H-I). The strain variation curves were superimposed and could rapidly return to its original form with full recovery during decrement of the load in a co-phasal way with the least hysteresis and energy loss. 50CC+H scaffolds also showed reversible stress-strain characteristics, with only a trace of hysteresis loop indicating inelastic energy residual after thermal treatment process. After 28 days incubation, a similar dynamic loading test at increasing cycles for over $2 \times 10^6$ times at 37.5°C was carried out; all scaffolds exhibited similar entropic elastic behavior confirming once more the stiffness softening effect of the scaffolds (Figure 3.6 J-L).
Figure 3.6 Stiffness softening of PUU-POSS scaffolds with a 50% infill density under cyclic tensile and compression after incubation at 37.5 °C for up to 28 days (n=2). (A-C) Dynamic tensile load in strain domain. (D-F) Dynamic tensile and compression loading profile of scaffolds at 0-200 cycles day 0 and after 28 days; (G-I) Dynamic compression loading profile of scaffolds at increasing cycles at day 0; (J-L) Dynamic compression loading profile of scaffolds at increasing cycles at day 28.

Temperature-dependent dynamic mechanical analysis (DMA) and wide-angle X-ray diffraction spectra at 37.5 °C scaffolds elucidated the association of unique phase transitions and viscoelasticity with the observed stiffness relaxation (Figure 3.7 A-C and Table 3.5). Unlike 50CC+H and 50RTC+H scaffolds, where DMA showed typical profiles of storage modulus ($E'$) and loss moduli ($E''$) as functions of temperature corresponding to the glass transition at ~35°C and rubbery plateau above room temperature, the storage modulus of 50CC scaffolds underwent a continuous decline over a wide range of temperatures above the glass transition. Furthermore, the abrupt peak in $E''$ previously seen around
the melting point at 45–50°C on DSC was replaced with an unusual wide tanδ “plateau” (Figure 3.7A). The prolonged viscoelastic behaviour observed in 50CC scaffolds implies a wide spectrum of relaxation times of chain segments in different length involved in the phase transition of crystal-to-amorphous rubber of the soft segments and local re-organisation of soft and hard segments. These sequential transitions from crystalline to random to quasi-random structures is proven through the disappearance of two sharp Bragg diffraction peaks at 2θ=20.0° and 23.4° in 50CC scaffolds early in incubation and a subsequent upshifting of two amorphous halo peaks centred at 17.9° and 21.3° to three with closer packing at 19.9°, 31.3° and 41.3° over 4 weeks (Figure 3.7 D-E). Regardless of the different initial stiffness of scaffold groups, all three scaffold types eventually “remembered” to self-organise back to a thermal dynamically stable rubber phase with a unique quasi-nanophase structure, demonstrating a delicate balance between entropic and energetic contributions as seen in Figure 3.7 D-F, and Table 3.6. This inverse assembling process did not cause unacceptable shape changes after 4 weeks, which we attribute to the low crystallinity and local interactions of hydrogen bonds. Regardless the initial stiffness produced at different thermal process conditions, as illustrated in Figure 3.7 G, this stiffness softening was driven by two stages of thermodynamic phase transition and local chain self-assembly: the 1st order phase transition due to melting of semicrystalline soft domain, followed by a low-dimensional and short-distance inverse self-assembly of the nanostructures towards a quasi-random nanophase crossing over a wide range of chain relaxation times.
Figure 3.7 Stiffness relaxation and corresponding changes of phase structure of 3D PUU nanohybrid scaffolds (50% infilled density) with different 3D-TIPS processing conditions after incubation at 37.5 °C for up to 28 days. (A-C) Dynamic mechanical properties as function of temperature at frequency of 1 Hz at day 0. (D-F) Wide-angle X-Ray diffraction (WAXD) spectra of the scaffolds on the different days of incubation (up to 28 days). (G) Schematic of phase transition of nanophase structure during stiffness softening of the scaffolds at day 0 and day 28.
### Table 3.5 Swelling ratio of PUU-POSS scaffolds before and after incubation

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Sample Size, (L×W×T, mm) (n=6)</th>
<th>Apparent Volume (mm$^3$)</th>
<th>Volume Swelling Ratio vs $V_{PVA}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% infill PVA preform (mould)</td>
<td>60.0×12.0×4.0</td>
<td>2880±4</td>
<td></td>
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<tr>
<td>50CC</td>
<td>Wet, as produced, RT</td>
<td>61.0×13.0×3.6</td>
<td>2854±9</td>
</tr>
<tr>
<td></td>
<td>In water at 37.5 °C for 21 days</td>
<td>62.0×14.0×4.0</td>
<td>3472±7</td>
</tr>
<tr>
<td>50CC+H</td>
<td>Wet, as produced, RT</td>
<td>59.7×11.3×3.5</td>
<td>2361±7</td>
</tr>
<tr>
<td></td>
<td>50CC+H in water at 37.5 °C for 21 days</td>
<td>60.9×12.7×3.6</td>
<td>2784±7</td>
</tr>
<tr>
<td>50RTC+H</td>
<td>Wet, as produced, RT</td>
<td>58.9×12.7×3.9</td>
<td>2917±13</td>
</tr>
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<td></td>
<td>50RTC+H in water at 37.5 °C for 21 days</td>
<td>60×13.3×4.2</td>
<td>3351±9</td>
</tr>
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### Table 3.6 Hysteresis values (i.e., energy loss) of the various scaffolds during tensile cyclic loading in the strain (25%) domain before day 0 and after day 28 at increasing cycles.

<table>
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<tr>
<th>Type of test</th>
<th>Day</th>
<th>Hysteresis energy (J/m$^3$)</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
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<tbody>
<tr>
<td>Tensile</td>
<td>D0</td>
<td>0-200 cycles</td>
<td>257.7±27.4</td>
<td>42.8±8.5</td>
<td>14.2±5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000-1200 cycles</td>
<td>163.4±1.1</td>
<td>20.6±4.6</td>
<td>14.3±2.9</td>
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<tr>
<td></td>
<td></td>
<td>10,000-10,200 cycles</td>
<td>23.8±8.4</td>
<td>13.6±3.2</td>
<td>13.8±4.2</td>
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<tr>
<td></td>
<td></td>
<td>200,000-200,200 cycles</td>
<td>15.3±4.9</td>
<td>7.6±3.3</td>
<td>12.1±3.5</td>
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<tr>
<td>D28</td>
<td>0-200 cycles</td>
<td>31.2±5.7</td>
<td>16.8±3.9</td>
<td>12.3±4.1</td>
<td></td>
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<tr>
<td></td>
<td>1000-1200 cycles</td>
<td>17.4±5.4</td>
<td>12.5±5.3</td>
<td>9.9±2.5</td>
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<tr>
<td></td>
<td>10,000-10,200 cycles</td>
<td>12.2±4.6</td>
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<td>200,000-200,200 cycles</td>
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<td>Compression</td>
<td>0-200 cycles</td>
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</tr>
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Table 3.7 Evolution of X-Ray diffraction peaks of the scaffolds with stiffness softening in vitro in Figure 3.4K-M. Degree of crystallinity (Dc, %), d-spacing (d, Å)
<table>
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<tr>
<th></th>
<th>50Cu:H</th>
<th>50Cu:H</th>
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<tr>
<td>Sharp peak 2</td>
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<tr>
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Table 3.8 Physical and mechanical properties of PUU-POSS scaffolds (50% infill) before and after incubation at body temperature for 28 days.

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<tr>
<th></th>
<th>3D-TIPS scaffold, 50% infill</th>
<th>Scaffold Densit y, kg/m³</th>
<th>Total Porosity, 100%</th>
<th>Tensile Modulus, MPa (Tensile)</th>
<th>Ultimate Tensile Strength, MPa (Tensile)</th>
<th>Ultimate Tensile Strain, % (Tensile)</th>
<th>Toughness, J. m⁻³×10⁴ (Tensile)</th>
<th>Compresssion Strength @25%, MPa</th>
<th>Compresssion Modulus @25%, MPa</th>
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<td></td>
<td>Day 0</td>
<td>35.71 ±4.70</td>
<td>96.90 ±0.30</td>
<td>0.98 ±0.14</td>
<td>1.33 ±0.09</td>
<td>179 ±8</td>
<td>136 ±22</td>
<td>0.33 ±0.02</td>
<td>0.51 ±0.08</td>
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<td>Day 28</td>
<td>29.30 ±3.90</td>
<td>97.40 ±0.30</td>
<td>0.45 ±0.08</td>
<td>0.77 ±0.15</td>
<td>230 ±13</td>
<td>114 ±19</td>
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<td>Day 0</td>
<td>45.30 ±5.30</td>
<td>96.10 ±0.40</td>
<td>0.53 ±0.02</td>
<td>0.76 ±0.05</td>
<td>235 ±18</td>
<td>112 ±27</td>
<td>0.22 ±0.04</td>
<td>0.27 ±0.03</td>
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<tr>
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<td>Day 28</td>
<td>38.50 ±4.90</td>
<td>96.60 ±0.40</td>
<td>0.39 ±0.09</td>
<td>0.72 ±0.12</td>
<td>240 ±17</td>
<td>109 ±13</td>
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<td>0.44 ±0.06</td>
<td>0.67 ±0.03</td>
<td>145 ±14</td>
<td>145 ±11</td>
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<td>149 ±19</td>
<td>145 ±19</td>
<td>0.17 ±0.02</td>
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3.3.4 Effect of stiffness softening of the scaffolds on HDF Cell Proliferation in vitro

Due to distinct differences of the surface and porous structure between the 3D printed scaffolds and standard 2D cell culture, it is imperative to optimize the cell seeding density on the 3D scaffolds. The metabolic activity of primary human dermal fibroblast (HDF) cells with five different densities seeded onto the 50CC scaffolds were tested over time for 10 days (Figure 3.8A). Cell metabolic activity measured by alamarBlue® assay showed that one day after seeding with the same density, 3×10⁴ cells/cm³, there are significantly fewer cells attached to the 50CC scaffolds compared to standard 2D TCP control. Many cells fell or migrated to adhere to the bottom tissue culture plastic because of a large surface area and many regular micro-pores (200-400 μm) of the 3D scaffolds compared to 2D TCP. There is a small increase of cell metabolism until day 7 and then slightly increased by day 14. By increasing 3 times the cell density, 9×10⁴ cells/cm³, the cell attachment in day 1 was improved and metabolic activity of the cell increased over 14 days, following the similar linear profile as that of TCP. By further increasing the cell density to 2.7×10⁵ cells/cm³, the cell attachment at day 1 was significantly increased and the metabolic activity of the cells increased up to day 5 and then reached a plateau at cell confluence. The alamarBlue® reading for the scaffolds with the two highest seeding densities, 8.1×10⁵ cells/cm³ and 2.43×10⁶ cells/cm³, show a similar trend. The highest cell density rendered the highest cell attachment on the scaffolds, but the early cell confluence and an overall decrease in cellular activity after day 3 suggested that the cells either stopped to grow or cell metabolism decreased after
confluence. Based on these results, an optimized cell density of $9 \times 10^4$ cells/cm$^3$ in 500 µl of cell culture medium ($2.5 \times 10^4$ cells/scaffold) was then used for further experiments.

Using optimized cell seeding densities, cellular metabolic activity on the elastomer scaffolds with three different processing conditions were measured over time. The metabolic activity of seeded HDF cells increased over time on the elastomer scaffolds with different stiffness (Figure 3.8 B). 50CC scaffolds showed superior cell proliferation at all time points compared to 50CC+H and 50RTC+H groups, with significance at days 10 ($p<0.001$), unlike control cells seeded on 2D TCP where metabolic activity levelled off after day 10, probably due to limited surface area after confluence. Proliferation and migration, as measured by increasing DNA concentrations in HDF cells and deposition of extracellular acid-soluble collagen (types I-V) by HDF cells cultured on 3D scaffolds with time, showed that the 50CC scaffold resulted in the highest values at all time points respectively (Figure 3.8 C-D). Live/dead staining corroborated this increase in proliferation with live HDF cell attachment observed within the 3D interconnected structure of the 50CC, 50CC+H, and 50RTC+H scaffolds (Figure 3.9 - 3.11). Cells were observed migrating towards the inner digital porous network of 50CC scaffolds from day 3 (Figure 3.9 A-A2), covering more than half of the scanned area by day 7 (Figure 3.9 B-B2). By day 10, live cells were visualised throughout the scaffolds' interior, filling the large infill generated pores (Figure 3.9 C-C2).

Figure 3.8 Cell seeding density test and cellular proliferation of human dermal fibroblasts (HDF) on PUU-POSS scaffolds. (A) Metabolic activity of HDF on PUU-POSS scaffolds. 50CC, and the collagen coated tissue culture plastic (TCP) control using different seeding density over 14 days, measured by the alamarBlue® fluorescence assay. TCP seeding density is $3 \times 10^4$ cells/cm$^3$. (B-D) HDF cell proliferation on scaffolds subjected to different phase separation conditions measured by (B)
Confocal microscopy with counterstaining, SEM imaging illustrating cell morphology and histological analysis of fixed cell-scaffold specimens provided further evidence of HDF proliferation, self-organisation and HDF cells-scaffold interactions during the stiffness relaxation. On immunofluorescence with HDFs stained green for actin and blue for nuclei, cell penetration and attachment were clearly seen in all three PUU-POSS scaffold groups at days 3 and 7 (Figure 3.12). Cells self-organised within the scaffold pores at different depths (showing different colours), confirming cell penetration through to the scaffold construct core (Figure 3.12 E-E1), and well-orientated blue and green interference fringes (Figure 3.12 D-D2 and 3.12 E-E2) and circular patterns (Figure 3.12 D-D2) suggested alignment and self-organisation preferences at different local layers throughout the scaffold. 3D-reconstructed confocal images (Figure 3.12 A3-F3) corroborated the 50CC scaffolds’ superior cell attachment and penetration, intra-scaffold proliferation and cellular organisation throughout the period of stiffness relaxation compared to 50CC+H and 50RTC+H (Figure 3.12 A2-C2). Histological staining of a lateral cross section of fixed cell-scaffold complexes on day 10 confirmed cell attachment and proliferation to the inside of all scaffold groups, with cells aggregating around the open infill pore edges created through 3D printing (Figure 3.13 A-F). HDF morphology on the surface across the scaffold groups was showed using SEM (Figure 3.13 G-L), with flatter cell bodies and long actin spindles on day 3 of the early proliferation and with increased cell coverage on the surface of all three scaffolds on day 7 (highlighted in blue). It is of note that the high density of cell proliferation was visualised on the scaffolds from the 3D reconstructed fluorescent images (Figure 3.9 - 3.11), which was compiled from 428 images scanned throughout 3 mm thickness of the scaffold at 7 um/Z-step (as described in Section 3.25). The H&E stained cell clusters within the thin layer of cross section (4 µm in thickness) of the scaffold (Figure 3.13 A-F) and the SEM morphology of the cells on the top surface of the scaffold (Figure 3.13 G-L) provided supporting evidence, in consistence with the results of the cell migration and proliferation throughout the scaffolds by 3D confocal microscopy.

There was a clear association of the 50CC scaffolds stiffness relaxation and porous surface topography with cell adhesion and metabolic activity compared to the other ‘softer’ 50CC+H and 50RTC+H processing conditions. Unlike 50RTC+H scaffolds, it is hypothesized that the high porosity and hierarchical porous structure in the 50CC scaffolds (Table 3.2 and 3.3) allowed fast diffusion of nutrients and oxygen from the cell culture media to the cells, whilst its high initial stiffness and sequential stiffness relaxation may have further accelerated cellular attachment, metabolic activity and proliferation in comparison to the softer 50CC+H scaffolds.
Figure 3.9 Live-dead staining confocal microscopy of cellular proliferation of human dermal fibroblasts on PUU-POSS scaffolds, 50CC by live (green)-dead (red) staining confocal microscopy. (A-C) (×10 objective lens) and (A1-C1) (×20 objective lens) HDF proliferation on 50CC scaffolds at day 3, day 7 and day 10. (A2-C2) 3D reconstructions of fluorescent light intensity by confocal microscopy (×10 objective lens).
**Figure 3.10** Live-dead staining confocal microscopy of cellular proliferation of human dermal fibroblasts on PUU-POSS scaffolds, 50CC+H by live (green)-dead (red) confocal microscopy. (A-C) (×10 objective lens) and (A1-C1) (×20 objective lens) HDF proliferation on 50CC scaffolds at day 3, day 7 and day 10. (A2-C2) 3D reconstructions of fluorescent light intensity by confocal microscopy (×10 objective lens).
Figure 3.11 Live-dead staining confocal microscopy of cellular proliferation of human dermal fibroblasts on PUU-POSS scaffolds, 50RTC+H by live (green)-dead (red) staining confocal microscopy. (A-C) (×10 objective lens) and (A1-C1) (×20 objective lens) HDF proliferation on 50CC scaffolds at day 3, day 7 and day 10. (A2-C2) 3D reconstructions of fluorescent light intensity by confocal microscopy (×10 objective lens).
Figure 3.12 HDF cell proliferation on PUU-POSS scaffolds at different stiffness relaxation stages. Confocal microscopy images showing stained HDF actin fibers (green) with counterstained (blue) nuclei at (A-C) day 3, (D-F) day 7, (G1-I1) day 10, and (G2-I2) day 14; (A-F) ×10 objective lens; (A1-F1) ×20 objective lens; (A2-F2) close-up images from A1-F1 (~×45 objective lens); (A3-F3) 3D reconstructions of fluorescent light intensity by confocal microscopy (~×10 objective lens).
Figure 3.13 Histological and scanning electron microscopic (SEM) analysis of HDF cells on PUU-POSS scaffolds at different stage of stiffness relaxation. (A-C) Fixed sections showing HDF cell proliferation around the scaffold pores in plane at day 10, stained with H&E (×10 objective lens). (D-F) are close-up images from (A-C) (~×20 objective lens). (G-L) SEM images showing HDF cell attachment and morphology at day 3 (G-I) and day 7 (J-L). (A, D, G, J) 50CC; (B, E, H, K) 50CC+H; (C, F, I, L) 50RTC+H.

3.4 Discussion

It has been demonstrated the manufacture of a body-temperature-responsive bespoke tissue scaffold through a reliable, versatile and cost-effective 3D-TIPS indirect printing technique. Large and complex 3D scaffolds with a wide range of structures and properties were fabricated from PUU-POSS nanohybrid elastomer solution confined within a scalable 3D-printed interconnected PVA preform network, with dual
control of multi-level phase-separation and polymer crystallinity. Thermal control of PUU-POSS solution coagulation and micro-phase separation of polymer chains within the preform network micro-channels produced 3D scaffolds with identical and uniform macroscopic dimensions and polymer content but with highly variable micro-/nano-structures, biomechanical and cellular properties. Scaffolds (CC group) subjected to temperatures below freezing during fabrication developed semi-crystalline structures, more nano-pores and less shrinkage, rendering them stronger and stiffer. Scaffolds (RTC+H group) processed at room temperature were softer and hyperelastic with higher strain and toughness values, due to the formation of quasi-random nanophase structures with packed hard domains acting as physical crosslinking points within the continuous amorphous rubber. Digital control of PUU-POSS scaffold infill density from 80 to 30% can also be manipulated to further widen the range of scaffold specification options without altering chemistry or using crosslinking, allowing specifications of each to best match the properties of various native tissues.

Variations in the infill density were found to affect the mechanical properties of the scaffolds, with overall greater tensile and compressive stiffness and strength for higher infill densities (Figure 3.4-3.6). In addition, 3D-TIPS of the scaffolds (CC group) exhibited overall the highest tensile and compressive mechanical properties compared to the CC+H and RTC+H groups with thermal treatment regardless of the infill density. This can be explained in terms of an ordered crystal lattice structure in the CC scaffolds and more crystal formation within the soft segment chains of their PUU, compared to a quasi-random nanophase amorphous structure in the RTC+H and CC+H group with little crystalline domains left due to temperature variations during the coagulation process and post thermal treatment. Isothermal relaxation of the static tensile and compression properties (Figure 3.4-3.5) and dynamic cyclic tests (Figure 3.5) of the scaffolds further proved the different levels of stiffness softening of the scaffolds at body temperature, depending on their initial stiffness produced at different thermal process conditions. Such viscoelastic behaviour is attributed to the phase transition of melting crystalline domains and subsequent self-assembling of nanophase structure, with the CC scaffold exhibiting the greatest reduction (more than 70%) by 28 days.

This manufacturing versatility lends bio-mimicry to our products, for example approximating characteristics of human soft tissues. For instance, femoral non-mineralized and mineralized fibrocartilage exhibit compressive modulus in the range 0.55 - 0.80 MPa and 0.20 - 0.60 MPa respectively [196] for the femoral and tibial condyles. The compressive modulus of the human ear cartilage is in the range 0.06 - 2.10 MPa [197-200], whilst human costal cartilage three-point bending values are 5 - 7 MPa [201]. Bone ECM has a high non-mineralized collagen content with compressive stiffness values of 0.1 - 1 MPa depending on stage of osteoid mineralization [202, 203]. Prior to their stiffness relaxation, the fabricated scaffolds reached maximum compression modulus and strength of 0.80 - 0.10 MPa upon the infill density and coagulation processing, making them worthy of some consideration for cartilage tissue engineering applications such as joint capsule articular or ear cartilage, and potentially, non-load bearing bone engineering. Furthermore, thermoresponsive scaffolds offered an interesting opportunity as an in vitro and in vivo model system to explore their effect of stiffness relaxation on host cells (in Chapter 4, 5 and 6).
Ease of handling during surgery is an essential feature of any implant, and the dynamic nature of tissues should be replicated for enhanced tissue regeneration. Here again, there is room for further work, since 3D-TIPS scaffolds exhibited stiffer mechanical support under typical surgical conditions, whilst relaxing their stiffness during typical remodelling physiological conditions. The major relaxation event occurred between 7- and 14-days post-incubation for 50CC and 50CC+H groups (Figure 3.5), whilst at 28 day’s incubation all groups relaxed to their intrinsic elasticity, reaching fairly uniform values. The stiffness relaxation of the scaffolds was also accelerated by cyclic loading tests after over 200,000 cycles under 25% larger compression strain within three days (Figure 3.6).

The most intriguing property of these scaffolds, that of stiffness softening, is driven by the reverse self-assembly of phase transition of 3D ordered crystalline structure into quasi-random nanostructures of soft and hard segments at body temperature, which imparts the unique and desirable dynamic mechanical properties of stiffness relaxation with unnoticeable shape change. The small volume changes, due to the relaxation of ordered chain packing at the melting point of soft segments within the uniform interconnected porous network contributed to the stability of the scaffold shape with little deformation. This is different from shape memory of polymers with a large strain (entropic elasticity) involved in the initial temporal shape. Regardless the initial stiffness generated at different thermal process conditions, 3D-TIPS PUU-POSS scaffolds ‘remembered’ to relax to its intrinsic hyperelastic rubber phase around the melting temperature of the soft segments.

The semi-crystalline rigid PUU-POSS constructs, coupled with stiffness relaxation and 3D-interconnected nano-pores produced in our study, resulted in the greatest HDF cells-scaffold attachment, penetration, migration and proliferation throughout these rigid scaffolds. Scaffold elasticity and viscoelasticity were revealed to be strong influences in various types of cell behaviours in well-established 2D models of variable stiffness polymers [55]. However, the stiffness changes in those 2D models were often coupled with the change of molecular structure due to the use of crosslinking agents. This study reveals for the first time that strong HDF cells proliferation remains during scaffold stiffness relaxation without changes in matrix molecular structure.

Like native ECM, these 3D scaffolds appear to offer a complex structure that is organized at several magnification scales. The scaffold shape and digitally controlled large pores (100-400 µm) provided specific guides and boundaries for HDF cells growth, offering the framework for local cells adhesion and proliferation. The cells demonstrated permeation into scaffolds, penetrating through the 3D digitally printed interconnected pores. The disordered, rough and nano-porous surface topography created by phase separation, may have contributed to improving cell adhesion and metabolic activity. The high continuous surface area within the scaffolds of 50CC (58.5 m²/g) and 50CC+H (24.5 m²/g, Table 3.3) allowed fast diffusion of nutrients from the cell culture media to the cells. The in vitro study suggests that the level of porosity (50% infill density) and size of the pores (100-400 µm) of the scaffolds are adequate to facilitate the attachment, migration and proliferation of HDF cells on the 3D-TIPS scaffolds.

As aforementioned, despite several reports on manufacturing polymeric scaffold by combination of 3D printing and TIPS [159, 160], dual-level regulation of porosity and stiffness relaxation, using design to
control macro-to-micro scale changes and thermally controlled phase-separation to govern micro- to nano-scale structure and properties, has not been described. The technique allows use of a wide range of biofunctional and unprintable polymers and their composites, and permits flexibility in the architecture of fabricated constructs, tunability not presently achievable through current direct printing techniques or phase-separation alone. New and existing polymer solutions, especially high molecular weight, and their nanocomposite solutions can be directly shaped into intricate and bespoke patient-specific 3D scaffolds through a one-stop processing technique. This low-cost method with short-lead time shows capability of shaping these highly permeable and lightweight 3D constructs, from a variety of unprintable degradable, non-degradable polymers and nanocomposite. This is especially the case for expensive new biomaterials with a low quantity, as well as the biomaterials that have been extensively studied at various preclinical, clinical and proven stages.

Controllable scalability of 3D-TIPS technique produced an array of constructs from the same polymer solution, without altering chemistry or using crosslinking, allowing specifications of each to best match the properties of various native tissues. The responses of various types of cell-lines to these tunable scaffolds were under investigation with positive results, including human bone-marrow derived mesenchymal stem cells (in Chapter 4), human bronchial fibroblast (in Chapter 5), human bronchial epithelial cells (in Chapter 5), and mouse embryonic dermal fibroblasts (in the Chapter 6). This is a potentially highly desirable property for aiding biological tissue remodelling following surgical tissue reconstruction. The improved cell viability on the 3D-TIPS scaffolds represents a promising technique platform for the development of biological responsive tissue-engineered implants, devices and surgical robotics, with matched dynamic mechanical properties to suit cell-lines, tissues and organs.

3.5 Conclusion

In this Chapter, 3D-TIPS technique opens up the use of a wide range of bio-functional and previously unprintable polymers. Their nanocomposites and permits tunability in the architecture and stiffness of fabricated constructs not previously achievable via direct printing techniques or phase-separation alone. The potential for improved cell growth and bio-responsiveness of scaffolds in the postoperative healing period, due to stiffness relaxation, represents a promising technique platform for the development of biological responsive tissue-engineered implants, devices and surgical robotics, with matched dynamic mechanical properties to suit dynamic cell-lines, tissues and organs.
Chapter 4

Stiffness Softening of 3D-TIPS Elastomer Nanohybrid Scaffolds Regulating Chondrogenesis and Osteogenesis of Human Bone-Marrow Mesenchymal Stem Cells

4.1 Introduction

Human mesenchymal stem cells (MSCs) reside in tissues of varying stiffness and participate in tissue regeneration with their stiffness changing as the repairing tissue matures and remodels [204]. This suggests that stem cells in different states may respond differentially as they commit to a specific fate, and apart from sensing their current environment, stem cells are affected by memory of their mechanical history [205-207]. For instance, human bone-marrow derived mesenchymal stem cells (hBM-MSC) cultured on soft or stiff hydrogels and later transferred to substrates of opposite stiffness [207] switched from neurogenic differentiation towards osteogenic lineages, whilst MSCs that were transferred from stiff to soft substrates retained elevated osteogenesis markers.

Stimulus-responsive polymers change their structure, physical or chemical properties, in response to various external stimuli. Such responsive materials, mainly hydrogels, have been developed recently [202, 208] and applied to study temporal stiffening [205, 209, 210] and softening [206, 211] effects of the substrate on cells both in 2D and 3D cell cultures. For example, Guvendering and Burdich studied the effects of a hydrogel whose stiffness increased by light-mediated crosslinking in the presence of hBM-MSCs [212]; stiffening at an earlier stage leads to osteogenic differentiation, while stiffening at later times leads to an equally mixed osteogenic/adipogenic fate. Yang et al. investigated the use of ultraviolet radiation to soften a hydrogel through photo-degradation and the mechanical memory of hBM-MSCs during culture [206] based on the expression of the transcriptional activator Yes-associated protein (YAP). YAP was found to be activated in the nucleus of MSCs cultured on stiff substrates while it deactivated and relocated to the cytoplasm when cells were cultured on soft substrates instead. They demonstrated that by softening a stiff hydrogel at different culturing times, YAP-transit to the nucleus affected cellular “memory”. Abdeen et al. used a magneto-activated gel to study the effects of varying
substrate elasticity on hMSCs differentiation [205]. They showed how a magnetic field could modulate cell spreading and cytoskeletal tension via changes in the matrix stiffness, with an impact on the secretion of proangiogenic molecules by hMSCs. Cells exhibited osteogenic activity when cultured on soft substrates whose matrix stiffness was magnetically increased at later time points. Independently controlling both composition and elasticity of a substrate is difficult, and also limits the range of stiffness achieved through changing a hydrogel’s physico-chemistry [213]. In addition, these settings present several limitations, from short-term stability issues to problems with cytocompatibility [212, 214]. Moreover, nearly all above strategies lead to an increased dynamic stiffening of the substrate; little work has been performed on the effect of stiffness softening of cell-seeded substrates.

The non-degradable poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) nanohybrid scaffolds with stiffness softening properties at body temperature towards their intrinsic elasticity through inverse self-assembling have been recently developed using 3D printing guided thermal induced phase transition (3D-TIPS) (in Chapter 3). Herein, the effects of stiffness softening on differentiation of hBM-MSCs with reference to chondrogenic and osteogenic lineages have been systematically investigated.

4.2 Materials and methods

4.2.1 Fabrication and characterization of the scaffolds

4.2.1.1 Design and fabrication of elastomer nanohybrid scaffolds using an indirect printing 3D-TIPS technique

PUU-POSS elastomer nanohybrid solution was synthesized as previously described in Section 2.2.1 Chapter 2. PUU-POSS scaffolds were then produced by 3D-TIPS, a 3D thermal induced phase-separation (TIPS) of the polymeric solution within 3D printed poly(vinyl alcohol) (PVA) preforms, used as water soluble sacrificial moulds as described in Section 2.2.2 Chapter 2. Three scaffold groups with different mechanical properties and porous structures were produced by the 3D-TIPS technique at different thermal processing conditions (shown in Section 3.2.1 Chapter 3 and Table 3.1): Cryo-Coagulation (CC), Cryo-coagulation + Heating (CC+H), and Room Temperature Coagulation + Heating (RTC+H).

4.2.1.2 Physic characterization of the scaffolds via wettability

General captive bubble contact angle measurement descriptions were previously referred in Section 2.3.4.4 Chapter 2.

4.2.2 In vitro experiments

4.2.2.1 Protein adsorption (BCA assay)

Detailed descriptions were previously referred in Section 2.3.4 Chapter 2.
4.2.2.2 Expansion, cell seeding and differentiation of hBM-MSCs

Human bone-marrow derived mesenchymal stem cells (hBM-MSCs; Sciencell™, California, USA) were subcultured and expanded with mesenchymal stem cell medium (MSCM; Sciencell™, California, USA) in a T75 flask (see Figure 4.1). Detailed description was shown in Section 2.4.1 Chapter 2.

Figure 4.1 Schematics of hBM-MSCs culture, expansion, seeding and differentiation towards the chondrogenic and osteogenic lineages.

Chondrogenic differentiation was carried out on day 1 post-seeding. Briefly, MSCM was discarded and replaced with 1 ml of mesenchymal stem cell chondrogenic differentiation medium (MCDM) supplemented with 10% of mesenchymal stem cell chondrogenic differentiation supplement (MCDS) (Sciencell™, California, USA), 5% penicillin/streptomycin (Sciencell™, California, USA), and 10 ng/ml TGF-β3, (differentiation inducer) (Miltenyi Biotec Ltd., Surrey, UK). Medium was replaced every third day of culture according to the manufacturer’s instructions throughout a 4-week culture. PUU-POSS scaffolds (n=4) were collected at days 1, 7, 14, 21, 28 and 35.

Osteogenic differentiation was carried out on day 1 post-seeding. Briefly, MSCM was discarded and replaced with 1 ml of mesenchymal stem cell osteogenic differentiation medium (MODM) supplemented with 10% of mesenchymal stem cell osteogenic supplement (MODS) (Sciencell™, California, USA). Medium was replaced every second day of culture according to the manufacturer’s instructions throughout a 4-week culture. PUU-POSS scaffolds (n=4) were collected at days 1, 7, 14, 21 and 28.

Human dermal fibroblast (HDF) cells served as negative control of differentiation. Spheroids were used as positive control of differentiation: 1×10⁶ cells were centrifuged, and the cell pellet placed in the incubator at 37°C in a humidified atmosphere of 95% air and 5% CO₂; spheroids formed within 24 h.
Spheroids were used as a positive control to demonstrate the success of the differentiation protocol and were compared with the differentiation of hBM-MSCs on the fabricated scaffolds. In addition, human femoral head (HFH) was used as positive control for comparison to native mature osteo/chondrocytes. HFH was collected from patients (with written consent) undergoing total knee replacement surgery at the Royal National Orthopaedic Hospital, approved by the UK Health Research Authority (REC reference: 15/LO/2052).

Cell-laden scaffolds \((n=4)\) after \textit{in vitro} differentiation were also subjected to compressive mechanical testing following the same procedure as previously referred in Section 2.3.4.5 Chapter 2.

4.2.2.3 Morphology of cell-seeded scaffolds

Cell-laden scaffolds \((n=2)\) after osteogenesis and chondrogenesis were stored in a vial containing primary fixative solution, 4% paraformaldehyde (PFA) in PBS and 2% glutaraldehyde in a 0.1 M cacodylate buffer at pH 7.4 and 4°C over-night. Detailed description can be referred to in Section 2.4.8 Chapter 2.

4.2.2.4 Immunohistochemistry analysis

Cell-laden scaffolds were washed in diH\(_2\)O before being fixed in 4% PFA in PBS. The samples were permeabilised with 0.1% triton X-100 (Sigma, UK) in PBS and non-specific binding of primary antibody was inhibited by incubation with 3% BSA (Sigma, UK) in PBS. They were then incubated with either 1/100 mouse anti-Collagen1 polyclonal antibody (Abcam, Cambridge, UK), 1/100 rabbit anti-Collagen 2 polyclonal antibody (Abcam, Cambridge, UK), 1/100 rabbit anti-SOX9 polyclonal antibody (Abcam, Cambridge, UK) or 1/100 mouse monoclonal anti-Aggrecan (Abcam, UK) in 3% BSA in PBS for 2 h at room temperature. Adjacent sections were incubated with Immunoglobulin G (IgG) antibody as negative controls. A 1/500 goat anti-mouse Alexafluor®-594, 1/500 goat anti-rabbit Alexafluor®-647 secondary antibody or 1/500 goat anti-rabbit Alexafluor®-555 secondary antibody (Molecular Probes, UK) in 1% BSA in PBS was added for 1 h at room temperature followed by counterstaining with phalloidin Alexafluor®-488 (Sigma, UK), respectively, to counterstain for F-actin. Finally, samples were stained with DAPI (Sigma, UK). 1/500 OsteoSense 680 EX (NEV10020EX; perkinelmer, USA) was used for staining as marker of calcium.

An alizarin red staining assay was performed according to the manufacturer’s instructions to look at calcium deposition. Briefly, after 21 days in osteogenic medium, cell-laden polymer discs were fixed with 4 % PFA in PBS, washed twice with deionized water (diH\(_2\)O) and stained with 1% Alizarin red S (ARS, pH 4.2) for 20 min at room temperature. Excess stain was washed away with two changes of diH\(_2\)O.

Images were taken using a confocal microscope (Leica TCS SP8vis) with ×10 and ×20 water immersion objective lens as previously referred in Section 2.4.7 Chapter 2.
4.2.2.5 Quantitative analysis of sulfated glycosaminoglycans (sGAG)

The ratio of sGAGs/DNA was quantified on the various PUU-POSS scaffold groups \((n=6)\) over a 4-week period by means of a Blyscan™ sulphated glycosaminoglycan assay (Biocolor Ltd.; Antrim, UK). General descriptions were previously referred in Section 2.3.6 Chapter 2.

4.2.2.6 Gene expression via quantitative reverse transcriptase polymerase chain reaction (qPCR) analysis

General descriptions were previously referred in Section 2.4.10 Chapter 2. Genes related to chondrogenic differentiation \((SOX9, COL2A1, COLX and ACAN)\) and osteogenic differentiation \((ALP, COL1A1, RUNX2, SPP1, BGLAP, SP7)\) were analyzed by qPCR analysis over a period of 28 and 21 days respectively. Expression of the house-keeping gene \(GAPDH\) served for normalization \([215]\). Genes related to chondrogenic differentiation (shown in Table 4.1) and osteogenic differentiation (shown in Table 4.2) were analyzed by qPCR analysis for 28 and 21 days respectively.

Table 4.1 List of primers used for qPCR (chondrogenesis)

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<th>Gene</th>
<th>Primer sequence (sense/antisense)</th>
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<td>SOX9</td>
<td>5’-GCCTTTTTGTCCATCCCTTTTTTC-3’ 5’-GTCCTTGGGGTTTCTTGCTGATGTA-3’</td>
<td>64.6 65.3</td>
</tr>
<tr>
<td>COL2A1</td>
<td>5’-ACCTCAGGCCCTCCCCATCATG-3’ 5’-ACATCAGGTCAGGTCCCGATCAG-3’</td>
<td>62.0 62.6</td>
</tr>
<tr>
<td>COLX</td>
<td>5’-TGAAAGGGACTCATGTTGTTGAGTGG-3’ 5’-ACTCAGAATTGAGCTGAGAATC-3’</td>
<td>60.5 60.4</td>
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<tr>
<td>ACAN</td>
<td>5’-TGAGGAGGCTGGAACAGTACC-3’ 5’-GGAGGGTCAATTGGGAGGAACA-3’</td>
<td>61.0 62.3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-TGATGACATCAAGAGGTGTGAG-3’ 5’-TCCITTGGAGGCCATGGG-3’</td>
<td>60.0 60.0</td>
</tr>
</tbody>
</table>

Note: SOX9, transcription factor SOX9; COL2A1, collagen type II; COLX, collagen type X; ACAN, Aggrecan; GAPDH, glyceraldehyde phosphate dehydrogenase.
### Table 4.2 List of primers used for qPCR (osteogenesis)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (sense/antisense)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>5'-GCCTTTTTGTCCATCCCTTTTTTC-3' 5'-GTCCTTGGGTTCTTGCTGATGTA-3'</td>
<td>64.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65.3</td>
</tr>
<tr>
<td>COL1A1</td>
<td>5'-CGCTACTACGGGGCTGATG-3' 5'-GTCCTTGGGTTCTTGCTGATGTA-3'</td>
<td>62.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.6</td>
</tr>
<tr>
<td>RUNX2</td>
<td>5'-AGAGGTTACAGAGGACTGTTGGTT-3' 5'-GGTAGCTACTTGGGGAGGATTTGTG-3'</td>
<td>61.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.63</td>
</tr>
<tr>
<td>SPP1</td>
<td>5'-ACTTGGGAAGGGTCTGTGGGGCT-3' 5'-AGGCATCACCTGTGCCATACCA-3'</td>
<td>60.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60.4</td>
</tr>
<tr>
<td>BGLAP</td>
<td>5'-ATGAGAGCCCTCACACTCCTC-3' 5'-GCCGTAGAAGGCCGATAGGC-3'</td>
<td>61.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.3</td>
</tr>
<tr>
<td>SP7</td>
<td>5'-TGCACTCTCCCTGCGCCAGACCTC-3' 5'-AACGGGTCTCCCAAGGGGAGGAGGAG-3'</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60.0</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGATGACATCAAGAGGTTGTTGGAAG-3' 5'-TCCTTGGAGGCCATGTGGGCCC-3'</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60.0</td>
</tr>
</tbody>
</table>

**Note:** ALP, alkaline phosphatase; COL1A1, collagen type I; RUNX2, cbfa-1; SPP1, Osteopontin; BGLAP, Osteocalcin; SP7, Osteorix; GAPDH, glyceraldehyde phosphate dehydrogenase.

### 4.2.2.7 ELISA analysis

Enzyme-linked immunosorbent assay (ELISA) was used to detect chondrogenesis presence of Aggrecan, Collagen II and glycosaminoglycan (GAG) content in 3D scaffolds (n=3) within their cell culture medium at weeks 2, 3 and 4. Osteogenesis presence of Osteocalcin and Collagen I was also detected at weeks 1, 2 and 3. ELISA kits used were human COL2 ELISA kit (Abbexa; Cambridge UK), human GAGs ELISA kit (Abbexa; Cambridge UK), human Aggrecan ELISA (Abcam, UK), human osteocalcin quantikine ELISA Kit (R&D System, UK) and COL1 ELISA Kit (Antibodies-online, UK). Optical density was determined using a microplate reader (Anthos 2020 microplate reader; Biochrome Ltd, UK). All experiments were carried out in triplicate.
4.2.2.8 Histological analysis

Differentiated hBM-MSCs on the various PUU-POSS scaffold groups (n=2) (days 21 or 28) were fixed in 4% PFA in PBS. Briefly, cell-laden samples were embedded in paraffin wax and cut into 4 μm thick sections using a rotary microtome, Leica RM2235 (Leica Microsystem Ltd., Milton Keynes, UK). Slide sections were then deparaffinized and stained with haematoxylin and eosin (H&E) stains, to indicate gross cell morphology.

Cartilage-like ECM production was investigated using Alcian Blue (A-Blue) staining for polysaccharide indication (e.g. glycosaminoglycans), Masson’s trichrome (MT) staining for collagen indication, and antibody collagen II (COL2) staining for collagen II production. Osteocyte-like ECM production was investigated using Alizarin Red S (ARS) staining for calcium indication, and antibody collagen I (COL1) staining for collagen I production.

4.2.2.9 Element detection by EDX analysis

General descriptions were previously referred in Section 2.3.4.3 Chapter 2. A human femoral head bone-cartilage joint was used as control.

4.2.2.10 Quantitative analysis of mineralization

The area of mineralization, as stained with Alizarin Red (ARed-Q, Sciencell™, California, USA), was quantified using ImageJ (NIH, USA) of bright field images. In brief, images were converted to a binary grayscale, a threshold was set to highlight only stained areas and the area was measured. In addition, the amount of ARS extracted from the samples was quantified by the method of Gregory et al. [216]. Absorbance was measured at 520 nm with a microplate reader (Anthos 2020 microplate reader; Biochrome Ltd, UK).

4.2.2.11 Quantitative measurement of ALP activity

Alkaline Phosphatase (ALP) activity was measured using stable p-nitrophenol phosphate substrate by Alkaline Phosphatase Assay Kit (Colorimetric) (Merck, Millipore, USA). At each time point over a 3-week period, culture medium was removed by decantation and cells were washed with PBS and harvested in 1 ml universal ALP buffer (Merck, Millipore, USA). Cells were sonicated twice for 20 secs and centrifuged at 900×g (centrifugal force) for 5 min at 4°C. ALP activity in the supernatants was determined following addition of p-nitrophenyl phosphate substrate and the reaction was stopped using 100 µl of 0.1 N NaOH. The optical density was measured at 405 nm using a microplate reader (Spectra Max Plus 384 MK3; Thermo, UK). The ALP activity was calculated from a standard curve after normalization to total protein content, which was measured using the Bradford protein assay kit (Pierce, Rockford, USA). ALP experiments were repeated twice with n=5 for each substrate.
4.2.3 Statistical analysis

Statistical analysis of the results was performed using Graph-Pad Prism 6 (GraphPad Software San Diego, USA). Statistical significance was calculated by one-way (for analyzing one independent variables) or two-way (for comparisons across more than two independent variables) analysis of variance (ANOVA) using Tukey’s post hoc test, or two-tailed unpaired Student’s t tests (for parametric data, when comparing data between two groups). A value of p<0.05 was considered statistically significant. Distribution testing showed that all data was parametrically distributed.

4.3 Results

4.3.1 Stiffness softening effect on proliferation of hBM-MSCs

Human bone-marrow derived mesenchymal stem cells (hBM-MSCs) were seeded on the scaffolds, and their ability to attach and proliferate were assessed over a 14-day period (Figure 4.2-4.5). The 50CC and 50CC+H scaffolds showed a lower contact angle (Figure 4.3 A) and greater amount of protein adsorption (Figure 4.2 B) (p-value non-significant between them) compared to the 50RTC+H group (p<0.01), which may be attributed to the aforementioned hierarchical porous structures and resulting in a large surface area of the scaffolds (i.e. CC and CC+H) by cryo-3D-TIPS process (Figure 4.2, and Chapter 3 Figure 3.2 D1-F2, Figure 3.3 , and Table 3.3). The static contact angle measured is determined by the surface chemistry and the surface roughness of the membrane. PUU-POSS is a hydrophobic polymer in nature. Uniform micro- to nano porous structure on the surface of 50CC and 50CC+H appeared to act as capillary to absorb water, thus reducing the contact angle. The smaller pores of 50CC, the higher their capillary effect contributed to faster water absorption, thus decreasing the contact angle with decreasing pore sizes and increasing protein adsorption [217]. hBM-MSCs were found to be initially more metabolically active and proliferate faster (p<0.01) on softer 50CC+H scaffolds compared to the rigid 50CC group; however, following cellular viability and proliferation assays after a 10-day period, non-significant differences could be observed as the stiffness softening was occurring (Figure 4.2 C-D). The morphology of hBM-MSCs showed flat cell bodies and long actin spindles on all three scaffolds (Figure 4.2 E-G). Confocal microscopy at days 1 and 10 (Figure 4.3-4.5) confirmed cellular activity within 50CC, 50CC+H, and 50RTC+H group scaffolds as seen by immunofluorescent staining, and 3D reconstructions of fluorescent intensity demonstrated constant increase in cell density across the full thickness of the scaffold.
Figure 4.2 Cellular proliferation of hBM-MSCs on the various 50% infill 3D printed nanohybrid elastomer scaffolds during stiffness softening. (A) Contact angle; (B) Protein adsorption; (C) alamarBlue® fluorescence assay; (D) Total DNA analysis (n=6); (E-G) SEM images showing cell attachment at day 5 for (E) 50CC, (F) 50CC+H, and (G) 50RTC+H. **p<0.01; ***p<0.001.
Figure 4.3 Cellular proliferation of hBM-MSCs on 50CC PUU-POSS scaffolds. hBM-MSCs proliferation on scaffolds subjected to different phase separation conditions measured (A1, B1, C1; A3, B3, C3) (×10 objective lens) and (A2, B2, C2; A4, B4, C4) (×20 objective lens), hBM-MSCs proliferation on 50CC scaffolds with cell culture course as demonstrated through live (green)-dead (red) staining (A1, A2, B1, B2, C1, and C2) and actin fibers (green) with nuclei counterstained (blue) staining (A3, A4, B3, B4, C3, and C4) by confocal microscopy at day 1, day 5 and day 10. (A5, B5, C5) 3D reconstructions of fluorescent light intensity by confocal microscopy.
Figure 4.4 Cellular proliferation of hBM-MSCs on 50CC+H PUU-POSS scaffolds. hBM-MSCs proliferation on scaffolds subjected to different phase separation conditions measured (A1, B1, C1; A3, B3, C3) (×10 objective lens) and (A2, B2, C2; A4, B4, C4) (×20 objective lens), hBM-MSCs proliferation on 50CC+H scaffolds with cell culture course as demonstrated through live (green)-dead (red) staining (A1, A2, B1, B2, C1, and C2) and actin fibers (green) with nuclei counterstained (blue) staining (A3, A4, B3, B4, C3, and C4) by confocal microscopy at day 1, day 5 and day 10. (A5, B5, C5) 3D reconstructions of fluorescent light intensity by confocal microscopy.
Figure 4.5 Cellular proliferation of hBM-MSCs on 50RTC+H PUU-POSS scaffolds. hBM-MSCs proliferation on scaffolds subjected to different phase separation conditions measured (A1, B1, C1; A3, B3, C3) (×10 objective lens) and (A2, B2, C2; A4, B4, C4) (×20 objective lens), hBM-MSCs proliferation on 50RTC+H scaffolds with cell culture course as demonstrated through live (green)-dead (red) staining (A1, A2, B1, B2, C1, and C2) and actin fibers (green) with nuclei counterstained (blue) staining (A3, A4, B3, B4, C3, and C4) by confocal microscopy at day 1, day 5 and day 10. (A5, B5, C5) 3D reconstructions of fluorescent light intensity by confocal microscopy.
4.3.2 Stiffness softening effect on *in vitro* chondrogenesis of hBM-MSCs

hBM-MSCs were cultured with chondrogenic medium for differentiation towards the chondrogenic lineage, and RNA was collected over a 4-week period. Chondrocyte-like mesenchymal cells are presented by strong positivity for Collagen II (green) and Aggrecan (red) deposition on the 50CC+H group at day 28 as seen under confocal microscopy, followed by the CC scaffold with reduced Aggrecan deposition and the least on the 50RTC+H sample (Figure 4.6 A-F). The gene expression of cartilage associated ECM formation was quantified by qPCR. Gene expression of chondrogenic associated genes ACAN, SOX9, COL2A1 and COLX increased with culture time in all scaffold groups (Figure 4.6 G-K). Although all groups featured stimulated chondrogenic differentiation, results suggested higher chondrogenic associated gene expression in 50CC+H scaffolds (p<0.05) compared to 50CC and 50RTC+H groups. In fact, non-significant differences for all genes tested on the 50CC+H scaffold were observed at week 4 when compared to human femoral head (HFH) used as control (Figure 4.6 K). In addition, the amount of glycosaminoglycan production as sGAG/DNA (Figure 4.6 L) increased over time within all scaffold groups; in particular, sGAG/DNA level reached and maintained the highest at week 4 for the 50CC+H group, significantly higher (p<0.01), compared to the rest of the groups and the spheroids as positive control. Compared to HFH, non-significant differences were found at week 4 with respect to the 50CC+H scaffold. It is also noted that the difference of associated gene expression between 50CC and 50CC+H was gradually reduced during stiffness relaxation.
Figure 4.6 Chondrogenic differentiation on 50% infill elastomer nanohybrid scaffolds during stiffness softening. (A-F) Confocal microscopy of hBM-MSC under chondrogenic conditions at two different magnifications: chondrogenic differentiation after 4 weeks for (A, D) 50CC, (B, E) 50CC+H and (C, F) 50RTC+H showing Collagen II (green), Aggrecan (red) and cell nuclei (blue). (G-J) Chondrogenic differentiation of hBM-MSCs quantified by qPCR during a 4-week period: (G) chondrogenic SOX9 (transcription factor SOX9) expression; (H) chondrogenic ACAN (Aggrecan) expression; (I)
chondrogenic COL2A1 (Collagen II) expression; and (J) chondrogenic COLX (Collagen X) expression. (K) Comparison of quantitative analysis of gene expression at week 4 with human femoral head. (L) Quantitative analysis of synthesis of sulfated glycosaminoglycans μgGAG/μgDNA over a 4-week period compared to human femoral head. (M-O) ELISA analysis for presence of GAG, Aggrecan and Collagen II secretion to the medium at weeks 2, 3 and 4. The mean of triplicate wells is plotted, and the error bars represent SD. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; “-” represents the reference to which the p-value is compared.

Presence of GAG, Aggrecan and Collagen II released to the medium was further quantified by ELISA (Figure 4.6 M-O). The highest expression of GAG, Aggrecan and Collagen II was detected on the 50CC+H group at all day points compared to the rest of the groups (p<0.05), and non-significant differences were found at week 3 with respect to HFH suggesting full maturation on such scaffold. While the values quantified for the 50CC group were lower than those for the 50CC+H group, it was still significantly higher than the 50RTC+H group. Overall, this data suggests that the 50CC scaffold, and the 50CC+H group in particular, led to a more rapid effect in induction of chondrogenesis within hBM-MSCs. These results were further corroborated by histological staining at week 4 (Figure 4.7). Increased cellular penetration into the scaffold, alongside deposition of Collagen II and proteoglycan components of the extracellular matrix associated with chondrocytes were observed in 50CC+H and 50CC scaffold groups. Chondrocyte-like mesenchymal cells were embedded in a chondrocyte-like matrix presenting the typical cartilage pericellular lacunae more evident on the 50CC+H sample, strongly evident at higher magnification, relative to the spheroid control and human femoral head cartilage. EDX mapping of the scaffolds was also carried out to evaluate calcium and phosphorus distribution across the samples (Figure 4.8 A, Figure 4.9 A and Table 4.3). As expected for chondrogenesis, results confirmed low presence of both elements in the engineered tissue on all groups; in particular, 0.67 wt % P and 0.92 wt % Ca were quantified on the 50CC+H scaffold, similar to human femoral head cartilage (Figure 4.8 A, and Figure 4.9 C).
Figure 4.7 Histological analysis of chondrogenic differentiation on 50% infill elastomer nanohybrid scaffolds during stiffness relaxation. At week 4 in plane-sections for (A1-A4) 50CC, (B1-B4) 50CC+H, and (C1-C4) 50RTC+H; ×4 objective, stained with Hematoxylin and Eosin (H&E), Alcian Blue (A-Blue), Collagen II (COL2) and SOX9. At week 4 cross-section for (A5) 50CC, (B5) 50CC+H and (C5) 50RTC+H; H&E staining, ×4 objective. Chondrocyte morphology after 28 days hBM-MSCs differentiation, ×25 objective: H&E and A-Blue staining for (A1.1-A2.2) 50CC, (B1.1-B2.2) 50CC+H, (C1.1-C2.2) 50RTC+H scaffolds, (D1-D2) spheroid control, and (D1.1-D2.2) human femoral head cartilage.
**Figure 4.8** EDX quantification and compression mechanical properties of hBM-MSC seeded on the scaffolds after *in vitro* differentiation. (A-B) EDX analysis quantification of calcium and phosphorous after *in vitro* differentiation. (C-D) Mechanical compression modulus and strength after day 28 and day 35 chondrogenesis and day 21 and day 28 osteogenesis of hBM-MSCs compared to the cell-free scaffolds of day 0 and day 28 stiffness relaxation. *p<0.05; **p<0.01.
Figure 4.9 SEM and EDX imaging of hBM-MSCs cultured on the various scaffolds under chondrogenic and osteogenic conditions: (A) after 28 days chondrogenesis on 50CC, 50CC+H and 50RTC+H scaffolds; (B) after 21 days osteogenesis on 50CC, 50CC+H and 50RTC+H scaffolds; (C) human femoral head cartilage control; (D) human femoral head bone control. Scale bar 100 μm.
Table 4.3 EDX element analysis of scaffolds after day 28 chondrogenesis (human femoral head cartilage, HFH-C; weight %, wt %; atomic concentration%, at %)

<table>
<thead>
<tr>
<th>Element</th>
<th>HFH-C</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>wt %</td>
<td>at %</td>
<td>wt %</td>
<td>at %</td>
</tr>
<tr>
<td>C</td>
<td>65.6</td>
<td>79.7</td>
<td>69.5</td>
<td>83.5</td>
</tr>
<tr>
<td>O</td>
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<td>16.0</td>
<td>18.42</td>
<td>14.7</td>
</tr>
<tr>
<td>Na</td>
<td>2.3</td>
<td>1.5</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Si</td>
<td>1.3</td>
<td>1.2</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>P</td>
<td>0.6</td>
<td>0.3</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Ca</td>
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<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Au</td>
<td>11.4</td>
<td>0.8</td>
<td>8.4</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 4.4 EDX element analysis of the scaffolds after 21 days osteogenesis (human femoral head bone, HFH-B; weight %, wt %; atomic concentration %, at %)

<table>
<thead>
<tr>
<th>Element</th>
<th>HFH-B</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt %</td>
<td>at %</td>
<td>wt %</td>
<td>at %</td>
</tr>
<tr>
<td>C</td>
<td>77.6</td>
<td>88.9</td>
<td>83.6</td>
<td>91.4</td>
</tr>
<tr>
<td>O</td>
<td>8.2</td>
<td>7.1</td>
<td>7.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Na</td>
<td>0.7</td>
<td>0.4</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Si</td>
<td>1.4</td>
<td>0.7</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>P</td>
<td>0.8</td>
<td>0.4</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Ca</td>
<td>3.7</td>
<td>1.9</td>
<td>2.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Au</td>
<td>7.4</td>
<td>0.5</td>
<td>4.5</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

4.3.2 Stiffness softening effect on in vitro osteogenesis of hBM-MSCs

hBM-MSCs were also seeded on the three types of scaffolds and cultured with osteogenic differentiation.
medium to induce osteogenic differentiation, and RNA was collected over 4 weeks. Confocal microscopic images of immunofluorescent stained osteocyte-like mesenchymal cells cultured on the scaffolds for 21 days show strong positivity for collagen I (green), collagen II (blue) and calcium (red) deposition on the 50CC and 50CC+H scaffolds, followed by the 50RTC+H scaffold with a drastic reduction in the presence of calcium (Figure 4.10 A-F). More intriguingly, the intensity changes of overlapped green, blue and red at different focused layers of the 50CC and 50CC+H scaffolds provided evidence that osteogenic differentiation occurred within the scaffold frame at different depths, confirming cell penetration through to the scaffold construct core (Figure 4.10 J-O). The locally orientated mixed colourful interference fringes along the scaffold strut (Figure 4.10 A, B, D, E) and concentric patterns around the printed pore (Figure 4.5 A-B) suggested preference for the alignment collagen fibres and mineralization during ossification guided by 3D printed micro-channels, reminiscent of osteon-like microscopic columns in lamellar bone. Deposition of calcium in the scaffolds was corroborated by quantification of the calcium content area surrounding collagen (Figure 4.10 G), and further evaluated in terms of alkaline phosphatase (ALP) activity and Alizarin Red S (ARS) (Figure 4.10 H-I). ALP is an early-stage marker of osteogenic differentiation and precursor to calcium deposition. ALP activity gradually increased with time in all scaffold groups, but greater expression of ALP was observed in the 50CC sample (p<0.001) followed by the 50CC+H and 50RTC+H groups after 3 weeks of culture. On the other hand, calcium deposition is a late-stage marker of osteogenic differentiation and can be evaluated in terms of ARS. ARS gradually increased as well in all sample groups after 3 weeks. In particular, 50CC scaffolds stained with higher ARS content compared to the rest of the groups (p<0.0001).

The expression levels of several key regulators of osteogenic differentiation were also quantified using qPCR (Figure 4.10 J-O). The levels of all genes tested gradually increased with time in terms of osterix, alkaline phosphatase, Collagen I, alpha-1 and osteopontin expression. It is noted that the increase on the 50RTC+H scaffold was slowed down after 2 weeks of culture. Gene expression levels peaked at week 3, with successful osteogenic differentiation occurring within the first 21 days. 50CC scaffolds exhibited significantly (p<0.001) higher mean relative expression levels of all studied genes compared to the rest of the scaffold groups and the spheroid controls. Furthermore, osteogenic associated gene expression in the 50CC scaffold after 3 weeks differentiation was comparable to that of HFH used as control (p-values non-significant) (Figure 4.10 P). The 50RTC+H scaffold expressed the lowest at all day points. Presence of Collagen I and osteocalcin released into the medium was further quantified by ELISA (Figure 4.10 Q-R). In particular, the 50CC scaffold expressed the highest levels of Collagen I and osteocalcin over a 3-week period compared to the rest of the groups (p<0.001), and non-significant differences were quantified compared to HFH. These analyses were confirmed by histological staining in terms of Collagen I and ARS deposition (Figure 4.11) that highlighted differences between the samples. Stained 50RTC+H samples did not reveal much osteocyte-like matrix after 21 days of culture. Increased cellular penetration into the 50CC scaffold, alongside deposition of bone-like protein components of the extracellular matrix associated with osteogenesis were predominantly observed in the 50CC group compared to the rest of the groups relative to the spheroid control and human femoral head bone control. Furthermore, for verification of calcium, EDX mapping was used to confirm scaffold
calcium accumulations (Figure 4.8 B, Figure 4.9 B and Table 4.4). EDX mapping detected the presence of carbon, phosphorus, calcium and silicon in the deposited accumulations after 21 days. High levels of Ca on the 50CC scaffolds (2.66 wt %) were observed compared to those in the 50CC+H and 50RTC+H groups (1.48 wt % and 0.29 wt % respectively). For the human femoral head bone control (Figure 4.8 B, Figure 4.9 D), up to 3.72 wt % of Ca was quantified.

Cell-laden scaffolds were also evaluated after in vitro differentiation of hBM-MSCs in terms of their compression mechanical properties (Figure 4.8 C-D). Increase compression moduli were observed within the scaffold groups after chondrogenic and osteogenic differentiation compared to cell-free scaffolds at day 0 and day 28 post-incubation at 37.5°C. It is interesting that the compression modulus of 50CC groups finally exceeded 50CC+H group after extended 35-day chondrogenic differentiation (Figure 4.8 C).
Figure 4.10 Osteogenic differentiation on 50% infill elastomer nanohybrid scaffolds during stiffness softening. (A-F) Confocal microscopy of hBM-MSCs under osteogenic conditions at two different magnifications: osteogenic differentiation after 21 days for (A, D) 50CC, (B, E) 50CC+H, and (C, F)
50RTC+H showing Collagen 1 (green), Collagen II (blue) and calcium (red) deposition. Quantification of (G) calcium content surrounding Collagen, (H) alkaline phosphatase (ALP) activity and (I) Alizarin Red S (ARS) staining for 50CC, 50CC+H and 50RTC+H scaffolds after 21 days. (J-O) Osteogenesis differentiation of hBM-MSCs quantified by qPCR during a 4-week period: comparative analysis for (J) SP7 (Osteorix) expression; (K) ALP (alkaline phosphatase) expression; (L) COL1A1 (Collagen I) expression; (M) BGLAP (Osteocalcin) expression; (N) SPP1 (Osteopontin) expression and (O) RUNX2 (cbfa-1) expression. (P) Comparison of quantitative analysis of gene expression at week 4 with human femoral head. (Q-R) ELISA analysis for presence of Osteocalcin and Collagen I secretion at weeks 1, 2 and 3. The mean of triplicate wells is plotted, and the error bars represent SD. ***p<0.001; ****p<0.0001; referenced with respect to CC sample “-“.

**Figure 4.11** Histological analysis of osteogenic differentiation on 50% infill elastomer nanohybrid scaffolds during stiffness softening. At week 4 in plane-section for (A1-A3) 50CC, (B1-B3) 50CC+H; and (C1-C3) 50RTC+H; ×4 objective, stained with H&E, Collagen I (COL1) and Alizarin Red. At week 4 cross-section for (A4) 50CC, (B4) CC+H and (C4) 50RTC+H; ×1.5 objective, H&E staining. Osteocyte morphology after 21 days hBM-MSCs differentiation; ×25 objective, H&E and Alizarin Red staining for (A1.1-A2.2) 50CC, (B1.1-B2.2) 50CC+H, (C1.1-C2.2) 50RTC+H scaffolds, (D1-D2) spheroid control, and (D1.1-D2.2) human femoral head bone.
4.4 Discussion

The work was successfully applied to study the response and differentiation of stem cells in micro-niches. Starting stiffness and stiffness relaxation of the scaffolds can be modulated by the processing conditions (Cryo-Coagulation CC, Cryo-Coagulation + Heating CC+H, and Room Temperature Coagulation + Heating RTC+H) (in Chapter 3). This system had been used to study chondrogenesis and osteogenesis of hBM-MSCs during stiffness relaxation. The observed slow stiffness softening of the scaffolds at mammalian physiological body temperatures is reminiscent of the slow relaxation of soft native tissue and of post-surgical tissue healing. Understanding stem cell plasticity in vitro is important because hBM-MSCs are a widely used cell source for regenerative medicine clinical trials. Stem cells in different states respond differentially as they commit to a specific fate, and their physical environment critically influences this process [202].

The differentiation of hBM-MSCs showed different preference of the initial stiffness and stiffness softening, resulting in enhanced chondrogenesis on the softer scaffold and osteogenesis on the stiffer one. The systematic tests and analysis demonstrated that chondrogenesis was promoted to a greater extent on the 50CC+H scaffold (Figures 4.2 - 4.6), while the 50CC group promoted greater osteogenic differentiation instead (Figures 4.8, and 4.10) following exposure of the cells to chondrogenic and osteogenic media respectively. The subsequent stiffness relaxation period over 28 days stimulated more osteogenic activity than chondrogenesis of hBM-MSCs cultured in vitro. The low initial stiffness and porous structure of the 50CC+H scaffold appeared to promote more chondrogenesis of hBM-MSCs, compared to the rigid 50CC scaffold with similar porous structure and soft 50RTC+H one with less micro- and nano- pores. The differences associated with the gene expression between 50CC and 50CC+H became less significant by week 3 to 4, which may be attributed to enhanced cell attachment, migration and proliferation during the fast reduction of stiffness in the 50CC sample within the first two weeks of the chondrogenic culture. As more MSCs grew inside of the printed channels of the scaffold, their differentiation potential was presumed to be regulated by the cell-derived matrix microenvironment generated by earlier differentiated MSCs, which were guided by the scaffold substrate in the early first two weeks. In addition, 50RTC+H remained the lowest gene expression compared to 50CC and 50CC+H despite its initial low stiffness, indicating another influential role of micro- and nano-porous structure of the scaffolds on promoting chondrogenesis. Therefore, it is hypothesized that chondrogenesis of hBM-MSCs is well-maintained on 50CC+H scaffolds due to a combination of a softer matrix with hierarchical porous structure that provided its highest surface area to volume ratio and specific guides and boundaries for cells to initially attach and growth, stimulating cartilage-like integrin mediators and offering a suitable framework for local cell adhesion and proliferation on this type of scaffold (Figures 4.4). In turn, this may have promoted cell penetration and proliferation into the scaffold core throughout the stiffness relaxation period, with the evidence of superior compression modulus of 50CC-based constructs after an extended differentiation post stiffness softening (Figure 4.8C).

On the other hand, the high initial stiffness of the 50CC scaffold and a more profound stiffness relaxation effect induced more osteogenesis. In contrast to chondrogenesis, osteogenesis was mainly dependent
on the high initial stiffness and more profound relaxation effect provided by the 50CC porous scaffold with the evidence of the highest associated gene expression among the three types of scaffolds at all time points over 21 days (Figure 4.10). 3D printed microchannels appeared to guide the formation of the osteon-like lamellar structure during ossification with the highest mineralization in the 50CC (Figures 4.9 and Table 4.4). Although the scaffold already became softer within the first two weeks of relaxation, osteogenesis remained active and reached the peak on 21 to 28 days (Figure 4.8D), which may indicate the "mechanical memory" of the hBM-MSCs osteogenesis on rigid substrates. Integrins are known regulators of stem cell differentiation and an α2-integrin-ROCK-FAKERK1/2 axis was stimulated by the rigid matrices to promote RUNX2 activity, eventually leading to osteogenic fate [218]. In the osteogenic differentiation, it is envisaged that that MSCs were guided by the initial stiffness of the 50CC scaffold with enhanced local adhesion of mediated specific integrins on MSCs, activated RUNX2 expression through bone morphogenetic protein (BMP) pathway, leading to bone formation. During the subsequent stiffness softening, with more MSC migration, proliferation and differentiation into the scaffold in the first 10 days (Figure 4.2), the original mechanosensing of the MSC may have gradually shifted to de novo cell-derived matrix sensing in more physiologically relevant 3D microenvironment generated by MSCs themselves within the printed channels, demonstrating resilient cellular "mechanical memory" regardless the reduction of the stiffness. Besides, the difference of associated bone gene expression in 50CC+H and 50RTC+H remained despite non-significance, reflecting again the effect of porous structure. Clearly, the influence of the initial stiffness and stiffness softening on the MSC osteogenesis was predominant. Overall, these results appear to show that controlling stiffness and porosity thermally during manufacturing, along with an introduced substrate stiffness relaxation, significantly affects in vitro differentiation of hBM-MSCs.

Even when the matrix softened at body temperature (in Chapter 3 Figure 3.4-3.5) towards the intrinsic elasticity of the matrix, hBM-MSCs retained their "stemness commitment". This cellular "mechanical memory" effect has been previously reported, wherein mechanical properties of the substrate influenced cellular states in a manner that retained their specific activity while the matrix softened [205-207]. Analysis of the compressive mechanical properties of the scaffolds after in vitro hBM-MSC osteogenesis and chondrogenesis (Figure 4.8 C-D) demonstrated that a substantial component of the resulting stiffness may be attributed to cell-derived ECM [219, 220], especially in those scaffolds where increase hBM-MSCs differentiation occurred (i.e. 50CC for osteogenesis and 50CC+H for chondrogenesis). For the extended longer differentiation, the softest 50RTC+H scaffold group with less porous structure and dense smooth surface remained stable with no obvious changes within the period of tests and represented the least efficient differentiation. The soft and porous 50CC+H group showed a slight relaxation in the modulus with noticeable enhancements of the resting scaffold mechanical properties, attributed by the most efficient chondrogenesis guided via both porous structure and soft stiffness. Despite being relatively slow in the early stages of proliferation and chondrogenesis compared to 50CC+H, the outstanding mechanical performance of the 50CC group may be mainly attributed to stiffness relaxation. Therefore, porous structure and surface morphology may be the predominant regulator in the early stage of chondrogenesis differentiation. In osteogenic culture, the initial stiffness appeared to be the determining regulator for governing the osteogenic lineage in the beginning and
pronounce stiffness relaxation enhanced the efficiency of bone formation. Overall, only cryo-3D TIPS (50CC) scaffolds retained the full range of novel advantages in terms of proliferation and differentiation capabilities for hBM-MSCs, and this would be the preferred family member for further research towards possible clinical application.

4.5 Conclusions

This chapter study suggests that the transitional stiffness relaxation effect of a family of thermoresponsive stiffness scaffolds significantly influence hBM-MSCs proliferation and differentiation into the chondrogenic and osteogenic mesenchymal lineages. In the early stage of differentiation, stem cells seeded onto these scaffolds synthesize and promote deposition of cartilage-like and bone-forming proteins regulated by the initial stiffness and porous structure of the scaffold, and then appear to be favored by the subsequent stiffness softening effect exhibited by this intriguing family of materials. Stiffness softening enhances the efficiency of MSC growth and differentiation. As more MSCs grow within the 3D printed microchannel network, the differentiation signal pathway may switch from the initial substrate-mechanosensing to cell-derived matrix sensing in 3D microenvironment, accelerating the differentiation and leading to the maturation of the synthetic cartilage and bone tissue. Through their tunable stiffness and stiffness relaxation, these hierarchical porous scaffolds represent a promising platform for the development of smart and biological responsive tissue-engineered implants and devices, with matched dynamic mechanical properties to suit a variety of different dynamic cell-lines, tissues and organs.
Chapter 5

Engineering A Tracheobronchial Epithelial Model on 3D-TIPS Thermoresponsive Scaffolds

5.1 Introduction

The airway epithelium is of great interest since it acts as a primary protective barrier to the external environment defending against toxins, pollutants and pathogens, and maintains homeostasis by regulating the innate immune response [221, 222]. Despite numerous attempts by several groups, the creation of a synthetic airway has to date remained elusive, extremely high morbidity and mortality rates have been reported [12]. The reasons relate, in part, to the many integrated functions that the tracheobronchial epithelium is required to fulfill. These functions comprise: barrier integrity, mucus production, antimicrobial capability, and cilia motility [223]. Three main cell types - ciliated, secretory (primarily mucus-secreting goblet cells) and basal cells - support these functions. These cell types need to be re-capitulated in any synthetic construct and be arranged in a manner that permits the functions to be fulfilled. Three main factors as well have been identified as necessary for development towards airway tract replacements [223]; these are a basal lamina equivalent with collagen fibres for cell-cell interaction and polarization, mesenchymal fibroblasts, and the presence of an air-liquid interface system for proliferation and differentiation of bronchial epithelial cells [224].

Attempts have been made regarding this issue, but few long-term successes have been achieved [225-227]. Tani et al. developed a fibrous trachea-bronchial tissue architecture based on porous collagen scaffolds [228], but the barrier function was not properly maintained at the cell-material surface interface due to excessive permeability. O’Leary et al. had more success by using a collagen-hyaluronan bi-layer scaffold that facilitated the culture of bronchial epithelial cells on a 2D layer and their co-culture with lung fibroblasts [225]. Re-epithelialization of tracheal scaffolds remains one of the primary issues confronted in tracheal tissue engineering [13].

In this chapter, the creation of a biofunctionalized bespoke 3D-TIPS printed elastomer-collagen hybrid scaffold that permits the culture of bronchial epithelial cells (hBEpiCs) on monoculture and co-culture conditions with human bronchial fibroblasts (hBFs) or human bone-marrow derived mesenchymal stem cells (hBM-MSCs) were reported. The hybrid scaffold developed here is composed of interpenetrated
porous networks of a soft non-degradable poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) nanohybrid impregnated with collagen hydrogel. These scaffolds were incorporated as the sub-layer of an epithelial in vitro model co-cultured with either hBFs or hBM-MSCs. The feasibility of the hybrid scaffolds to support growth and differentiation of a bronchial epithelial cell line and its ability to support the co-culture with either hBFs or hBM-MSCs have been testified. The formation of an epithelial barrier, the structure and biofunctions of columnar ciliated epithelial and mucus-secreting goblet cells at the air-liquid interface of the synthetic tracheal tissue have been systematically characterized.

5.2 Materials and methods

5.2.1 Scaffold fabrication

Nanohybrid elastomer scaffolds based on a poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) composite were manufactured by an in-house 3D-TIPS approach as previously described in Section 3.2.1 Chapter 3. Briefly, a PUU-POSS elastomer solution was synthesized and injected into 3D printed poly(vinyl alcohol) (PVA) preforms (100 mm × 100 mm × 1.2 mm) with an orthogonal 50% infill density, used as water soluble negative sacrificial moulds. PUU-POSS was then cryo-coagulated within the preforms at −20°C for 24 h, followed by an iced water bath for another 24 h. Polymer discs (16 mm diameter, 1 mm thickness) were then cut from the PUU-POSS scaffolds.

Collagen hydrogel functionalized 3D-TIPS scaffolds (3D-TIPS+Collagen) were fabricated by infusing a collagen solution into the scaffold (16 mm diameter, 1 mm thickness) and subsequently compressed by using a process of Real Architecture for 3D Tissues (RAFT) [229]. Briefly, an 84% (v/v) rat tail collagen type I solution (First Link, UK) was mixed with 10% (v/v) 10× Eagle’s minimal essential medium (MEM) (Thermo Fisher, UK) until a homogenous yellow solution was obtained. Then, a 6% (v/v) NaOH/HEPES neutralizing solution (10M NaOH and 1M HEPES in a volume ratio of 0.198:1) was added and gently mixed. The mixture was held on ice prior to placing 1 ml of solution per scaffold on a 24-well plate. This was incubated for 15 min at 37°C to allow for collagen gelatinization prior to gentle wicking of water using hydrophilic porous absorbers (RAFT 3D systems; Lonza, UK), placed on top for 15 min at room temperature for liquid removal and pressed into the 3D-TIPS scaffold. Following absorption, the absorbers were removed, resulting in 1 mg/mm³ of collagen hydrogel/scaffold.

5.2.2 Cell culture

5.2.2.1 Cell selection and culture media

The human bronchial epithelial cell line (hBEpiCs) (ScienCell, California, USA) was used for monoculture and co-culture experiments. Cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, UK), mesenchymal stem cell medium (MSCM) (Sciencell™, California, USA) and bronchial epithelial cell medium (BEpiCM) (ScienCell, California, USA). Cells were used in fifth-passage (P5).
A human bronchial fibroblast cell (hBF) line (ScienCell, California, USA) was used (P5) for co-culture experiments. Cells were cultured in MEM (Sigma-Aldrich, UK) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, 26 mM sodium bicarbonate, 100 U/ml penicillin/streptomycin, and 1 mM sodium pyruvate (Sigma-Aldrich, UK), termed as EME. Cells were maintained at 37°C and 5% CO₂ in a humidified atmosphere.

A human bone-marrow derived mesenchymal stem cell (hBM-MSC) line (ScienCell; California, USA) was also used (P5) for co-culture experiments. These cells were cultured in mesenchymal stem cell medium kit (MSCM) (Sciencell™, California, USA) and maintained at 37°C and 5% CO₂ in a humidified atmosphere.

5.2.2.2 Seeding cells on 3D-TIPS transwell inserts

3D-TIPS and 3D-TIPS+Collagen scaffolds were assessed for their ability to support hBEpiCs growth and differentiation either as monoculture or co-culture with hBFs or hBM-MSCs under air-liquid interface (ALI) conditions (see Figure 5.1). Scaffolds were cut into 16 mm diameter discs (1 mm thickness) and clipped to CellCrown™ polycarbonate transwell housing for 24-well plate (Scaffdex Oy; Tampere, Finland).

For monoculture conditions, 1.2 ml of BEpiCM medium was added into each well, 3D scaffold inserts (3D-TIPS or 3D-TIPS+Collagen) were placed into the wells and the upper chamber was filled with 500 µl of Pneumacult™-ALI medium (STEMCELL technologies; Cambridge, UK). 5×10⁴ cells (2.5×10⁵ cells/cm²) were plated on to 3D scaffolds inserts. 3D scaffolds with cells were cultured under BEpiCM medium for 3 days to promote cells attachment. After day 3, scaffolds were “air-lifted” by replacing 1.2 ml of Pneumacult™-ALI medium to the basal chamber only while the upper chamber was empty to let cells be exposed to air. Finally, medium in the lower chamber was replaced every 2 days.

For co-culture conditions, 2×10⁴ cells (9×10⁴ cells/cm²) of either hBFs or hBM-MSCs were first plated onto 3D-TIPS+Collagen scaffold inserts and cultured under EME or MSCM respectively for 3 days to promote cell acclimatization and attachment. Subsequently, hBEpiCs were then seeded as described above. Samples were cultured in a 1:1 mixture of medium (BEpiCM-EME or BEpiCM-MSCM).

Polyethylene terephthalate (PET) transwell inserts were used as control. In a 24-well plate, 500 µl of BEpiCM was added to each well. A Falcon® PET filter membrane with 1.0 µm pore size (Becton Dickinson Labware; Clai, France) was used as positive control and placed into the wells. The upper chamber was then filled with 200 µl of BEpiCM medium. 1.8×10⁴ cells (1×10⁵ cells/cm²) were plated on to PET inserts to promote cell proliferation and growth until nearly confluent, according to the manufacturer’s instructions. PET inserts with adhered cells were cultured under Pneumacult™-ALI medium conditions for 3 days to promote cell attachment. After day 3, scaffolds were “air-lifted” by replacing 500 µl of Pneumacult™-ALI medium to the basal chamber only, while the upper chamber was left empty to allow the cells to be exposed to air.
5.2.3 *In vitro* analysis

5.2.3.1 Cellular proliferation

Media was replaced every two days, and the metabolic activity of cells was monitored on days 1, 3, 7, 10 and 14 to determine cell viability through alamarBlue® (AB) (Serotec Ltd., Kidlington, Oxford, UK) testing as detailed in Section 2.4.3 Chapter 2. At each day point, total DNA content was also quantified using a fluorescent Hoechst 33258 stain.

5.2.3.2 Immunofluorescence

Immunofluorescent staining was carried out to detect the presence of various markers of epithelial differentiation and functionality: MUC5AC, ZO-1, FOXJ1, Ki67, p63, vimentin, keratin 5, keratin 14, keratin 18, acetylated α-tubulin, e-cadherin and f-actin. Cell-laden scaffolds and cell inserts were washed in Dulbecco's Phosphate-Buffered Saline (DPBS; ThermoFisher, UK) and fixed in cold 4% PFA in PBS up to 18 h overnight. Samples were permeabilized with 0.1% (v/v) Triton-X 100 (Sigma-Aldrich, UK) for 15 min, rinsed with DPBS and blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, UK) in PBS solution for 30 min. Following further rinsing, they were then incubated with various fluorescent dyes and conjugated antibodies. A complete list is shown in Table 5.1. Finally, samples were mounted in Fluoroshield® with DAPI (Sigma-Aldrich, UK). Images were taken and analyzed using a confocal microscope (Leica Spvi8, Germany).
Table 5.1 List of fluorescent dyes and conjugated antibodies

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Dilution</th>
<th>Target</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst 33258</td>
<td>0.01 μg/ml</td>
<td>Nucleus</td>
<td>Sigma-Aldrich, Gillingham, UK</td>
</tr>
<tr>
<td>Alexa Fluor® 488 phalloidin</td>
<td>1 unit/200 μl</td>
<td>F-actin</td>
<td>Life-technologies, Paisley, UK</td>
</tr>
<tr>
<td>Rabbit anti- Ki-67 (primary antibody)</td>
<td>1 μg/ml</td>
<td>Nuclear localised proliferative cell marker</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Mouse anti – p63 (Primary antibody)</td>
<td>1 μg/ml</td>
<td>Basal proliferative epithelial cell marker</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Mouse anti- vimentinAlexa Fluor® 488</td>
<td>1 μg/ml</td>
<td>Class III intermediate filament; mesenchymal marker</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Mouse anti-Ecadherin Alexa Fluor® 488</td>
<td>1μg/50 μl</td>
<td>E-cadherin, adherens junction</td>
<td>BD Biosciences, Oxford, UK</td>
</tr>
<tr>
<td>Mouse antiacetylated α-tubulin</td>
<td>1 μg/ml</td>
<td>Microtubule, cilia structure</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Mouse anti- Mucin 5AC</td>
<td>1 μg/ml</td>
<td>Mucin 5AC, airway secretory mucous</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Rabbit anti-ZO-1 polyclonal antibody</td>
<td>1 μg/ml</td>
<td>Tight junction (TJ) protein marker</td>
<td>Molecular Probes, Invitrogen, UK</td>
</tr>
<tr>
<td>Rabbit anti - keratin 5 (Primary antibody)</td>
<td>1 μg/ml</td>
<td>Intracellular keratin 5, basal undifferentiated epithelial marker</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Rabbit anti - keratin 14 (Primary antibody)</td>
<td>1 μg/ml</td>
<td>Intracellular keratin 5, basal epithelial marker</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Mouse anti-keratin 18 (Primary antibody)</td>
<td>1 μg/ml</td>
<td>apical cell layer; intracellular keratin18, differentiated epithelial marker</td>
<td>Millipore, California, USA</td>
</tr>
<tr>
<td>Goat anti-mouse Alexa Fluor® 633 (Secondary antibody)</td>
<td>1 μg/ml</td>
<td>IgG</td>
<td>Life-technologies, Paisley, UK</td>
</tr>
</tbody>
</table>
5.2.3.3 Cell morphology by scanning electron microscope (SEM) and focused He-ion based microscope (He-ion FIB)

Cell-laden scaffolds were imaged using SEM/FIB to assess epithelial cell morphology and monolayer formation on the surface of the scaffold. Samples were fixed in 3% glutaraldehyde (Sigma-Aldrich, UK) for 1h at room temperature, dehydrated in a series of ethanol solutions and dried using supercritical carbon dioxide in a critical point dryer. Dry constructs were sputter-coated with gold (~8nm thickness) and imaged using Zeiss XB1540 FIB/SEM and Zeiss Orion NanoFab. Low magnification images were captured using XB1540 Fib/SEM at 1 kV and working distance of 3.5 mm, while high magnification images were taken using Orion NanoFab He-ion FIB at 25kV and working distance of about 7 mm.

5.2.3.4 Quantitative reverse-transcriptase polymerase chain reaction (qPCR)

**Table 5.2** List of primer assays used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>QuantiTect Primer Assays</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRT18</td>
<td>Hs_KRT18_1_SG QuantiTect Primer Assay</td>
<td>Differentiated cells</td>
</tr>
<tr>
<td>TJP1</td>
<td>Hs_TJP1_1_SG QuantiTect Primer Assay</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>Hs_MUC5AC_1_SG QuantiTect Primer Assay</td>
<td>Mucus production, goblet cell</td>
</tr>
<tr>
<td>FOXJ1</td>
<td>Hs_FOXJ1_1_SG QuantiTect Primer Assay</td>
<td>Epithelial cell ciliation</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs_GAPDH_1_SG QuantiTect Primer Assay</td>
<td>Housekeeping gene</td>
</tr>
</tbody>
</table>

Relative gene expression of epithelial cells seeded on the scaffolds (n=5 per group) was quantified using qPCR on weeks 1, 2 and 3. Detailed description was shown in Section 2.4.10. Chapter 2. Briefly, markers of epithelial primer assays (Qiagen, Valencia, CA) used are listed in Table 5.2, where
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an endogenous control “housekeeping gene” for all samples.

5.2.3.5 ELISA analysis

Enzyme-linked immunosorbent assay (ELISA) was used to detect presence of MU5AC, FOXJ1, ZO-1 and keratin 18 in 3D scaffolds within their cell culture medium at weeks 1, 2 and 3 (n=4 per group). ELISA kits used were MUC5AC (Abbexa, UK), TJP1 (ZO-1) (antibodies-online, UK), FOXJ1 (antibodies-online, UK), and keratin 18 (antibodies-online, UK). Optical density was determined using a microplate reader (Anthos 2020; Biochrome Ltd, UK). All experiments were carried out in triplicate according to the manufacturer’s protocol.

5.2.3.6 Histology

Cell-laden scaffolds (n=2) were fixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich, UK) in phosphate-buffered saline (PBS), embedded in paraffin wax and cut into 4 μm thick sections using a Leica RM2235 microtome (Leica Microsystems Ltd.; Milton Keynes, UK). Haematoxylin and eosin (H&E) staining was performed to examine gross cell location and morphology, and Alcian Blue-periodic acid Schiff (AB-PAS) staining to identify cells with mucosal substances and observe cell distribution and migration into the scaffolds. Images were taken using a digital slide scanner (Leica SCN400F, Germany) at ×40 magnification.

5.2.3.7 Transepithelial electrical resistance (TEER) measurement

The integrity of the epithelial barrier formed by hBEpiCs cultured on the 3D printed scaffolds was quantified by the measurement of TEER in monoculture and co-culture conditions. Prior to measurement of TEER using an EVOM voltohmmeter (World Precision Instruments; Stevenage, UK), cell culture medium was initially added to the apical compartment of the ALI cultures and samples were incubated for 1 h. Electrical resistance was measured using STX-2 chopstick electrodes (World Precision Instruments; Stevenage, UK) immediately upon removal of cells from the incubator. TEER was calculated by subtracting the resistance of a cell-free scaffold or insert and correcting for the surface area available for epithelial cell growth (0.39 cm²). To compare TEER values between groups following a plateau of the measurements [230], the average TEER values from day 14 were taken for each group and compared. The ohmic resistance of a blank (culture insert without cells) was measured in parallel. To obtain the sample resistance, the scaffold without cell of blank value was subtracted from the total resistance of the sample. The final unit area resistance (Ω/cm²) was calculated by multiplying the sample resistance by the effective area of the scaffold with cells.

5.2.3.8 Fluorescein isothiocyanate (FITC)-labeled dextran 70 (FD70) permeability assay

The integrity of the epithelial barrier formed by hBEpiCs on composite scaffolds was further assessed by analysis of FD70 paracellular transport through the cell layer [231]. Samples were initially washed and incubated with Hank’s buffered salt solution (HBSS; Sigma-Aldrich, UK) in both the apical and
basolateral compartments for 0.5 h. Subsequently, the buffer in the apical compartment was replaced with a 500 mg/ml solution of FITC-labelled dextran of an average molecular weight of 70 kDa (FD70), and the sampling from the basolateral compartment was performed every 20 min for 1 h to quantify transported drug. An equal volume of buffer was used to replace the removed volume of basolateral solution at each time point. Additionally, a sample of the initial apical FD70 content was taken for analysis and TEER measurements were performed before and after the experiment to validate that the barrier integrity was unaltered during the transport assay. The fluorescence of sampled time points was quantified by measuring excitation at 485 nm and emission at 535 nm. Fluorescence values were converted to concentration of FD70 using a standard curve and the apparent permeability coefficient (Papp) of FD70 was calculated:

$$Papp \text{ (cm/s) } = \frac{F}{(A \times C_0)} \quad (E5.1)$$

where F is flux (i.e. rate of change in cumulative mass transported), A is surface area of cell culture support, and C₀ is the initial concentration in donor chamber.

The theoretical pore radii of the cell layers was estimated by calculating the Renkin function [232]:

$$\frac{r_i}{r_p} = \left[1 - \left(\frac{r_i}{r_p}\right)^5\right]^2 \cdot [1 - 2.109 \left(\frac{r_i}{r_p}\right) + 2.09 \left(\frac{r_i}{r_p}\right)^3 - 0.95 \left(\frac{r_i}{r_p}\right)^5] \quad (E5.2)$$

where rᵢ is the molecular radius of the solute (nm) and rₚ is the theoretical pore radius (nm).

5.2.4 Data analysis

Statistical analysis of the results was performed using Graph-Pad Prism 6 (GraphPad Software San Diego, USA). Statistical significance was calculated by one-way (for analyzing one independent variables) or two-way (for comparisons across more than two independent variables) analysis of variance (ANOVA) using Tukey's post hoc test, or two-tailed unpaired Student t tests (for parametric data, when comparing data between two groups only). A value of p<0.05 was considered statistically significant.

5.3 Results

5.3.1 Epithelial cell growth on 3D-TIPS scaffolds

Cell seeding density onto a new scaffold is critical for the initial cell attachment and migration. For this reason, the optimization of the cell seeding process was initially performed using hBEpiCs at three different cell seeding densities (0.8×10⁴, 3×10⁴ and 5×10⁴ cells/scaffold) under monoculture conditions. Figure 5.2 A shows the cell seeding efficiency of hBEpiCs onto 3D-TIPS scaffolds over a 14-day period. Reduced metabolic activity was seen at 0.8×10⁴ and 3×10⁴ cells/scaffold during the cell culture. Despite a slight reduction on day 10, the optimal cellular viability at 5×10⁴ cells/scaffold remained increasing over time, which was chosen as the cell seeding parameter for further study. hBEpiCs cultured on collagen hydrogel functionalized composite scaffolds (3D-TIPS+Collagen) exhibited significantly greater
metabolic activity (Figure 5.2 B) and proliferation (Figure 5.2 C) in terms of alamarBlue® and total DNA assays over a 10-day period, compared to untreated 3D-TIPS scaffolds (p<0.001).

Immunofluorescence micrographs (Figure 5.2 D-H) show that a majority of hBEpiCs retained undifferentiated basal cells (keratin 5 [233]), meanwhile expressed markers for supporting epithelial differentiation (vimentin), stratified epithelium (p63 as a transcription factor [234]) and intercellular junctions (f-actin bundles) were observed on the periphery of hBEpiCs on all scaffolds at day 7, with p-value non-significant. In contrast, hBEpiCs on 3D-TIPS+Collagen scaffolds co-cultured with hBM-MSCs exhibited less percentage of stained mitotic cells (Ki-67 marker, Figure 5.2 D), indicating less DNA synthesis, compared to their co-culture with hBFs (p-value non-significant) and TIPS scaffold (p<0.01). More details of the cellular attachment and proliferation of hBEpiCs on day 7 were evaluated through confocal microscopy and SEM, as shown in Figure 5.3 A-D.

More lamellipodia (highlighted by white circles) were observed in close-up confocal microscopic images of basal undifferentiated cell marker (keratin 5) on the scaffolds with collagen hydrogel and co-cultured with hBFs and hBM-MSCs, compared to the untreated 3D-TIPS scaffolds, as shown in Figure 5.3 A1-D1. hBEpiCs also expressed more vimentin on those scaffolds, as shown in Figure 5.3 A2-D2. More actin filaments protruded to the edge of the cell membrane to propel cell attachment, movement and immigration by extending their lamellipodia around the edges of the cells to adhere to each other and to those functionalized scaffolds. SEM images in Figure 5.3 A3-D3 provide more information about cell morphology and the interface between the cells themselves and the scaffolds. hBEpiCs on the collagen functionalized and co-cultured scaffolds look flatted and connected to each other, even merged with blurred boundary on the hBM-MSCs co-culture, as opposed to the round shape with less actin filaments protruding and connections between the cells on the untreated scaffolds.
Figure 5.2 (A) Cellular viability of hBEpiCs seeded on 3D-TIPS scaffolds with different cell seeding densities, measured by alamarBlue® fluorescence assay for 14 days. A Falcon® PET filter membrane was used as positive control. (B) AlamarBlue fluorescence assay and (C) total DNA analysis of hBEpiCs monocultured on 3D-TIPS and 3D-TIPS+Collagen scaffolds. (D) Percentage cell count of stained cells on the various scaffolds. (E-H) Confocal microscopic images at day 7 showing hBEpiCs proliferation at each condition: (E) monoculture on untreated 3D-TIPS; (F) monoculture on 3D-TIPS+collagen; (G) co-culture with hBFs on 3D-TIPS+Collagen; (H) co-culture with hBM-MSCs on 3D-TIPS+Collagen. Cells were stained respectively for nuclei (blue), f-actin (green), p63 (magenta), keratin 5 (cyan), vimentin (yellow) and Ki-67 expression (red). ** p<0.01; ***p<0.001.
Figure 5.3 hBEpiCs proliferation on each conditional scaffold at day 7, imaged by confocal microscopy (keratin 5 (magenta) as basal undifferentiated cell marker, vimentin (yellow) intermediate filament marker, F-actin (green)) and SEM: (A) hBEpiCs monocultured on untreated 3D-TIPS scaffold; (B) hBEpiCs monocultured on 3D-TIPS+Collagen; (C) hBEpiCs co-cultured with hBFs on 3D-TIPS+Collagen; (D) hBEpiCs co-cultured with hBM-MSCs on 3D-TIPS+Collagen.

5.3.2 Epithelial ciliation in scaffold culture

The formation of motile cilia in scaffold culture was assessed through FOXJ1 (marker of epithelial cell ciliation) analysis of gene expression (Figure 5.4 A1) and ELISA analysis of FOXJ1 over a 3-week period (Figure 5.4 A2). hBEpiCs mono- and co-cultures on the 3D-TIPS+Collagen scaffolds showed
significant upregulation of FOXJ1 gene expression compared to the untreated scaffold and PET control for two weeks, indicating that 3D-TIPS+Collagen scaffolds promote more sustained increase in gene expression. More cilia were highlighted by acetylated alpha tubulin staining both in plane and cross-section of 3D-TIPS+Collagen scaffolds, compared to the untreated 3D-TIPS or control, and even much more extent in co-culture conditions at day 21 (Figure 5.4 B1-F1). A similar trend of formation of pseudostratified columnar epithelial cell arrays was also observed. Orion NanoFab He-ion FIB images (Figure 5.5 A-E) further revealed a dense and long pseudostratified columnar morphology on the composite and co-cultured scaffold (Figure 5.5 C1-E4), in contrast to the sparse dot-like morphology in cultures with PET cell inserts (Figure 5.5 A1-A4).

Cross-sectional images by He-ion beam (Figure 5.5 F) confirmed that an epithelial layer had covered the scaffold with more prominently longer and thicker ciliary structures formed on 3D-TIPS+Collagen scaffolds, especially when co-cultured with hBM-MSCs (Figure 5.5 G-H, and Table 5.3). The ciliary structures of hBEpiCs monoculture formed on 3D-TIPS+Collagen scaffolds were more prominent in length and diameter (Table 5.3) than those on untreated 3D-TIPS (7.01 ± 2.32 μm length and 0.09 ± 0.01 μm diameter vs. 3.80 ± 0.32 μm length and 0.08 ± 0.01 μm diameter, respectively) (Figure 5.5 G-H). Notably, the ciliary structures formed on 3D-TIPS+Collagen scaffolds seeded with hBEpiCs co-cultured with hBM-MSCs were longer and larger in diameter than those observed in co-culture with hBFs (9.78 ± 2.34 μm length and 0.17 ± 0.02 μm diameter vs. 8.18 ± 2.78 μm length and 0.11 ± 0.02 μm diameter respectively) (Figure 5.5 G-H). Together, the results demonstrated that the synergetic combination of a micro/nano-porous structure and collagen hydrogel within the matrix of the hybrid scaffold supported epithelial differentiation and that the co-cultured layer of hBFs and hBM-MSCs facilitated more efficient cilia formation on hBEpiCs.
Figure 5.4 (A) qPCR and ELISA analysis for (A1-A2) FOXJ1 as a marker for ciliation and (A3-A4) ZO-1 as a marker for tight junctions. (B-F) Confocal microscope images at day 21 showing (B1-F1) acetylated alpha tubulin and (B2-F2) ZO-1 protein expression, respectively for ciliation and adherens junction for the various scaffolds: (B) hBEpiCs monoculture on PET cell culture insert; (C) hBEpiCs monoculture on untreated 3D-TIPS; (D) hBEpiCs monoculture on 3D-TIPS+Collagen; (E) hBEpiCs co-culture with hBFs on 3D-TIPS+Collagen; (F) hBEpiCs co-culture with hBM-MSCs on 3D-TIPS+Collagen. Maximum intensity projections of ZO-1 (red) reconstructed from Z-stacks; cells counterstained for nuclei (blue) and E-cadherin (green), with cilia indicated by acetylated alpha tubulin (purple). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 5.5 (A-F) Orion NanoFab He-ion FIB images of hBEpiCs differentiation in an air-liquid interface (ALI) culture on various scaffolds at day 21: (A) monoculture on PET cell culture insert; (B) monoculture on untreated 3D-TIPS; (C) monoculture on 3D-TIPS+Collagen; (D) co-culture with hBFs on 3D-
TIPS+Collagen; (E) co-culture with hBM-MSCs on 3D-TIPS+Collagen; (F) cross-sectional slices showing a ciliated epithelium of hBEpiCs monoculture on 3D-TIPS+Collagen scaffolds. (G) Average mean cilia length \((n=20)\) and (H) average mean cilia diameter \((n=20)\) at day 21. **\(p<0.01\); ***\(p<0.001\); ****\(p<0.0001\).

Table 5.3 Morphology and quantification of ciliation development at day 21 \((n=20)\) on the various scaffolds

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Length ((\mu m))</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hBEpiCs PET insert monoculture</td>
<td>2.1 ((\pm 0.5))</td>
<td>69 ((\pm 10))</td>
</tr>
<tr>
<td>hBEpiCs 3D-TIPS monoculture</td>
<td>3.8 ((\pm 0.3))</td>
<td>79 ((\pm 10))</td>
</tr>
<tr>
<td>hBEpiCs 3D-TIPS+Collagen monoculture</td>
<td>7.0 ((\pm 2.3))</td>
<td>88 ((\pm 11))</td>
</tr>
<tr>
<td>3D-TIPS+Collagen hBEpiCs co-culture with hBFs</td>
<td>8.2 ((\pm 2.8))</td>
<td>109 ((\pm 14))</td>
</tr>
<tr>
<td>3D-TIPS+Collagen hBEpiCs co-culture with hBM-MSCs</td>
<td>10.8 ((\pm 2.3))</td>
<td>169 ((\pm 17))</td>
</tr>
</tbody>
</table>

5.3.3 Epithelial barrier formation and mucin expression on 3D-TIPS scaffold

The ability of the scaffolds to support hBEpiCs differentiation were also assessed by analysing the expression of the tight junction protein ZO-1 (also known as occludin-1) and adherens junction of associated protein, e-cadherin for binding adherens junctions as markers of bronchial epithelial barrier formation. hBEpiCs cultured on 3D-TIPS+Collagen scaffolds, either as monoculture or co-culture with hBFs or hBM-MSCs, exhibited an upregulation \((p<0.0001)\) of ZO-1 gene expression \((Figure 5.4 A3)\) compared to control or untreated 3D-TIPS scaffolds. After prolonged culture time over 3 weeks, hBEpiCs exhibited an increase of ZO-1 gene expression on 3D-TIPS+Collagen scaffolds. ELISA analysis of ZO-1 in the medium over a 3-week period also corroborated qPCR data \((Figure 5.4 A4)\); significantly \((p<0.05)\) higher ZO-1 was detected on the co-culture with hBM-MSCs compared to that with hBFs and the rest of the scaffolds. Formation of both tight junctions (ZO-1 in red, \(Figure 5.4 B2-F2\)) and adherens junctions (e-cadherin in green, \(Figure 5.6 B1-F1\)) were observed on the surface of all the scaffolds including PET control at day 21 through immunofluorescent images that captured the presence of the tight junction protein (ZO-1) and transmembrane protein (e-cadherin) between epithelial cells. These results indicate that adjacent epithelial cells formed intercellular junctions on the 3D-TIPS+Collagen scaffolds that restricted paracellular transport across the epithelium. Furthermore, cross-sectional images \((Figure 5.4 B1-F1\) to \(F2-1\) an \(Figure 5.6 E1-F1\) to \(F1-1\)) by confocal microscopy showed well-stratified tight junction columnar (ZO-1 in red, \(Figure 5.4 B1-1\) to \(F2-1\)), and adherens columnar junctions (e-cadherin in green, \(Figure 5.6 B1-1\) to \(F1-1\)) were vertically aligned under the epithelium for both co-culture conditions, compared to the monocultures. In particular, adherens junctions \((Figure 5.6 E1-1\) and \(F1-1\)) were well structured and connected in co-culture condition, and even extended into the supporting layer of hBM-MSCs. In contrast, e-cadherin appeared disoriented and disconnected underneath the epithelium in monoculture conditions. Therefore, these results all
together suggest that co-culture with hBM-MSCs enhanced the formation and maturation of epithelium barrier.

Mucin 5A (MUC5AC) is the gene associated with goblet cells that secrete mucin in the respiratory epithelium. Analysis performed by qPCR (Figure 5.6 A1) showed that MUC5AC gene expression is upregulated on 3D-TIPS+Collagen scaffolds, either as hBEpiCs monoculture or in co-culture with hBFs or hBM-MSCs (p<0.0001), compared to 3D-TIPS scaffolds and PET control following 2 weeks of cell culture. The increase of MUC5AC expression varied on different 3D-TIPS+Collagen scaffolds over time. Significant differences in gene expression were tested on 3D-TIPS+Collagen scaffolds seeded with hBEpiCs co-cultured with hBFs or hBM-MSCs at week 3 compared to monocultures (p<0.001). Those data indicate that 3D-TIPS+Collagen scaffolds promoted a sustained mucus-secreting epithelial phenotype that outperformed those in the conventional PET cell insert cultures. Similar trend was further confirmed by ELISA analysis of MUC5AC glycoprotein over a 3-week period (Figure 5.6 A2). Immunofluorescent staining of expressed MUC5AC glycoprotein at day 21 (red, Figure 5.6 B1-F1-1) corroborated the above quantitative analyses, showing more sustained mucus-secreting epithelial phenotype on the co-cultured samples, compared to those monoculture scaffolds, with significantly more on hBM-MSCs co-culture (Figure 6 F1-1). When hBEpiCs are co-cultured with either hBFs or hBM-MSCs on 3D-TIPS+Collagen scaffolds, a more sustained mucus-secreting epithelial phenotype was exhibited, compared to those monoculture scaffolds, with significant more MUC5AC expression on hBM-MSCs co-culture in consistence with the results from qPCR (p<0.05) and ELISA analyses (p<0.001). Overall, these results provide more clear evidence that hBEpiCs formed a functional epithelial barrier on the composite 3D-TIPS+Collagen scaffolds cultured at an air-liquid interface. Co-culture with hBM-MSCs significantly enhanced the stratification of epithelium by formation of more in-depth well connected adherens junctions and more goblet cells.
Figure 5.6 (A) qPCR and ELISA analysis for MUC5AC as a marker proteins of goblet cells of mucus production, and keratin 18 as marker protein of differentiated cell on 3D-TIPS scaffolds. (B-F) Confocal microscopic immunofluorescent stained images at day 21 showing (B1-F1) MUC5AC glycoprotein secretion; (B2-F2) intracellular keratin 14 and intracellular keratin 18; (B) hBEpiCs monoculture on PET cell culture insert; (C) hBEpiCs monoculture on untreated 3D-TIPS; (D) hBEpiCs monoculture on 3D-TIPS+Collagen; (E) hBEpiCs co-culture with hBFs on 3D-TIPS+Collagen; (F) hBEpiCs co-culture with hBM-MSCs on 3D-TIPS+Collagen. Maximum intensity projections of keratin 14 (yellow), keratin 18 (purple), and MUC5AC (red) reconstructed from z-stacks; cells counterstained for nuclei (blue) and e-catherin (green). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

5.3.4 Analysis of basal cell and differentiated cells

The expression of intracellular keratin 14 and intracellular keratin 18 indicated the organization of differentiated mitotically active basal epithelial cells and differentiated cells in the epithelium on the various scaffolds. qPCR and ELISA analysis showed that keratin 18 gene expression is upregulated on 3D-TIPS+Collagen scaffolds in both mono- and co-culture, compared to untreated control (Figure 5.6 A3-A4), and reached the highest on week 2 when co-cultured with hBM-MSCs (p<0.001, Figure 5.6 A4). Undifferentiated hBEpiCs at day 7 were stained with keratin 5 (Figure 5.2, and 5.3) and differentiated hBEpiCs at day 21 (Figure 5.6 B2-F2) stained with keratin 14 as marker of basal cells, and keratin 18 as marker of differentiated cells for distinguishing the basal and differentiated layers within the epithelium. As expected, at day 21 keratin 14 was mostly visible on the basal layer at cell-scaffold interface (Figure 5.6 B2-1 to F2-1), while keratin 18 on the upper layer of the epithelium on all groups (Figure 5.6 B2 to F2 and B2-2 to F2-2). More staining for both keratin markers were seen on 3D-TIPS+Collagen scaffolds compared to control or untreated 3D-TIPS. Cross-sectional images (Figure 5.6 B2-2 to F2-2) showed a thicker epithelium for co-culture conditions with hBM-MSCs where more keratin 18 appeared on the apical layer, indicating more basal cells differentiated into ciliated and secretory cells. Histological examination of 21-day cultured scaffolds (Figure 5.7 A-E) shows well-developed ciliated cells and muco-substance by Alcian blue-Periodic acid-Schiff (AB-PAS) (magenta at arrow head) on the scaffolds. An upper ciliated monolayer appeared more prominent on 3D-TIPS+Collagen scaffolds compared to the untreated and PET control, and the highest number of mucus-secreting cells observed on co-culture scaffolds with hBM-MSCs, resembling that of native bronchial epithelium. Further quantification of percentage of cell counts (Figure 5.7 F) stained by F-actin and AB-PAS also agreed with those by the qPCR and ELISA analyses above (Figures 5.4, and 5.6). The histological staining of the cross-sectional samples at day 21 confirmed the presence of an upper ciliated monolayer more prominent on 3D-TIPS+Collagen scaffolds compared to PET control or untreated 3D-TIPS, and the highest number of mucus-secreting cells observed on co-culture scaffolds with hBM-MSCs.
Figure 5.7 Hematoxylin & Eosin (H&E), Alcian blue-Periodic acid-Schiff (AB-PAS), and F-actin staining, respectively, at day 21 of cross-sections of differentiated hBEpiCs on different 3D-TIPS scaffolds: (A) hBEpiCs monoculture on PET cell culture insert; (B) hBEpiCs monoculture on untreated 3D-TIPS; (C) hBEpiCs monoculture on 3D-TIPS+Collagen; (D) hBEpiCs co-cultured with hBFs on 3D-TIPS+Collagen; (E) hBEpiCs co-cultured with hBM-MSCs on 3D-TIPS+Collagen. (F) Total percentage of cell count as seen by AB-PAS staining. **p<0.01; ***p<0.001; ****p<0.0001.
5.3.5 Epithelial barrier integrity in scaffold co-culture

Transepithelial electrical resistance (TEER) measurements (Figure 5.8 A-B) were used to confirm an effective barrier function of the differentiated epithelial layer on all the scaffolds with mean TEER values of 496 (±53), 972 (±211), 1110 (±320) and 1187 (±230) Ωcm² on day 14 for monoculture on 3D-TIPS, monoculture on 3D-TIPS+Collagen and co-cultures on 3D-TIPS+Collagen scaffolds with hBFs or hBM-MSCs respectively (Figure 5.8 B, and Table 5.4). TEER values increased dramatically in co-culture with hBFs or hBM-MSCs, although the values were not significantly different between them. The ability of the epithelial barrier to impede paracellular transport of FD70 was evaluated by Papp values in all scaffolds (Figure 5.8 C, and Table 5.5), showing that cells cultured on 3D-TIPS+Collagen were less permeable to solute flux than cells cultured on untreated 3D-TIPS. The Papp values and average molecular weight of the compound can be employed to determine the theoretical equivalent pore radii of the cell layer by equation E5.2; the epithelial layer in co-culture with hBFs or hBM-MSCs exhibited smaller pore radii of the cell layers (5.2 nm and 4.1 nm respectively) compared to monoculture conditions on PET, 3D-TIPS or 3D-TIPS+Collagen (65 nm, 62 nm and 13 nm respectively). Diffusion transport of FITC-dextran showed a dramatic reduction from the basolateral to apical on 3D-TIPS+Collagen with monoculture and co-culture conditions, in contrast to the diffusion from the apical to basal which remained fairly constant in Figure 5.8 D-E. Therefore, the decrease of the permeability for 3D-TIPS+Collagen scaffolds was mainly determined by the diffusion from the basolateral to apical, and the asymmetric diffusion through the cell layers for the two opposite diffusion directions was minimized through the better growth and maturation of the epithelium layer.
Figure 5.8 Transepithelial electrical resistance (TEER) of hBEpiCs cultured in monoculture and co-culture conditions with hBM-MSCs or hBFs for 14 days at an air-liquid interface on 0.385 cm² cell culture supports; plotted as a function of time from each conditional separate experiment at day 14: (A) transepithelial electrical resistance (TEER) (n=36); (B) average TEER values of epithelial cell barriers following plateau of electrical resistance (≥ day 14) (n=36); (C) apparent permeability coefficient (Papp) of fluorescein isothiocyanate-labelled dextran 70 (FITC-dextran) through the bronchial epithelial cell barrier at day 14 (n=36); (D) molecular diffusion of FITC-dextran on day 21, basolateral to apical vs.
apical to basolateral compartments \((n=6)\); (E) schematics showing apical to basolateral and basolateral to apical diffusion through the cell layer. *\(p<0.05\); **\(p<0.01\); ***\(p<0.001\); ****\(p<0.0001\).

### Table 5.4 Transepithelial electrical resistance (TEER) of the various scaffold types

<table>
<thead>
<tr>
<th>Day</th>
<th>PET insert monoculture</th>
<th>3D-TIPS monoculture</th>
<th>3D-TIPS+Collagen monoculture</th>
<th>3D-TIPS+Collagen co-culture with hBFs</th>
<th>3D-TIPS+Collagen co-culture with hBM-MSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>90 (±27)</td>
<td>56 (±31)</td>
<td>99 (±29)</td>
<td>399 (±193)</td>
<td>322 (±23)</td>
</tr>
<tr>
<td>5</td>
<td>140 (±59)</td>
<td>103 (±53)</td>
<td>231 (±111)</td>
<td>231 (±98)</td>
<td>554 (±243)</td>
</tr>
<tr>
<td>7</td>
<td>380 (±104)</td>
<td>89 (±53)</td>
<td>421 (±143)</td>
<td>721 (±543)</td>
<td>1372 (±251)</td>
</tr>
<tr>
<td>10</td>
<td>80 (±55)</td>
<td>511 (±53)</td>
<td>901 (±189)</td>
<td>1105 (±289)</td>
<td>1372 (±422)</td>
</tr>
<tr>
<td>14</td>
<td>470 (±130)</td>
<td>496 (±53)</td>
<td>972 (±211)</td>
<td>1110 (±320)</td>
<td>1187 (±230)</td>
</tr>
</tbody>
</table>

### Table 5.5 Apparent permeability coefficient at day 14 on the various scaffold types

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Papp (cm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET insert monoculture</td>
<td>(5 \times 10^{-4} (±1.1 \times 10^{-4}))</td>
</tr>
<tr>
<td>3D-TIPS monoculture</td>
<td>(4.3 \times 10^{-4} (±1.0 \times 10^{-4}))</td>
</tr>
<tr>
<td>3D TIPS+Collagen monoculture</td>
<td>(0.6 \times 10^{-4} (±0.3 \times 10^{-4}))</td>
</tr>
<tr>
<td>3D-TIPS+Collagen co-culture with hBFs</td>
<td>(0.2 \times 10^{-4} (±0.7 \times 10^{-5}))</td>
</tr>
<tr>
<td>3D-TIPS+Collagen co-culture with hBM-MSCs</td>
<td>(0.1 \times 10^{-4} (±0.5 \times 10^{-5}))</td>
</tr>
</tbody>
</table>

### 5.4 Discussion

The unique tunable initial stiffness and subsequent stiffness softening of the soft elastomer scaffolds at human body temperature induced by 3D-TIPS have also been characterized and demonstrated the potential to facilitate cell growth of HDFs (in Chapter 3) and modulate the differentiation of hBM-MSCs towards the chondrogenic and osteogenic cell lineages when cultured in differentiation media (in Chapter 4). In vivo study has further shown that the stiffness relaxation mechanism exhibited by 3D-TIPS 3D-printed scaffolds could modulate tissue ingrowth and promote good vascularization guided throughout the digitally defined network in rat models for up to 3 months with a reduced inflammatory response (in Chapter 6). These recent achievements have paved the way for the further development of more complex multi-layered tracheobronchial epithelium-like tissues in vitro based on these thermoresponsive elastomer nanohybrid scaffolds.

To improve hBEpICs viability, these scaffolds that served as a primary substrate were further functionalized by impregnating a collagen hydrogel within the interconnected porous network of the PUU-POSS synthetic elastomer, with proven benefit of collagen in respiratory epithelial culture [235,
The systematic study on hBEpiCs cell growth and differentiation on the functionalized composite scaffolds in both monoculture and co-culture conditions have elucidated the capacity of such scaffolds to support an epithelium bioprocess in vitro with formation of a tissue-like architecture, physiologically relevant to 3D human tracheobronchial biofunctions. The results provide coherent evidence of hBEpiCs' growth, expression of mucin and cilia and formation of an epithelial barrier with cell retention at the air-liquid interface supported by the scaffolds.

The interpenetrated hybrid network generated by infusing soluble collagen solution into the interconnected porous structure of synthetic PUU-POSS scaffold under compression and subsequent crosslinking has been proven to act as an ECM-like niche for hBEpiCs growth and differentiation. HBEpiCs could attach and grow on all the PUU-POSS scaffolds, however, the significant increase of hBEpiCs attachment and growth on 3D-TIPS+Collagen composite scaffolds compared to untreated ones and PET control (p<0.001, Figure 5.2-5.3) may be mainly attributed to the presence of collagen, correlating with the published literature on the beneficial effect of collagen in respiratory epithelial culture [10,19,32]. The response of cells to the external environment relies on the coordinated disassembly and assembly of cytoskeleton filamentous actin; this ability is critical for various cell functions including adhesion, wound healing, division, and locomotion [236].

The ability of the cells to differentiate and stratify to a functional epithelium on the scaffolds at the air-liquid interface is a further testimony of the functionalized 3D-TIPS scaffolds. 3D-TIPS+Collagen scaffolds were demonstrated to support the expression of cilia in hBEpiCs. Cilia are an important feature of a fully-functional tracheobronchial epithelium since they are an integral component of the respiratory tract that assists with removal of particulates and debris from the airway [237]. 3D-TIPS+Collagen scaffolds were demonstrated to support the expression of cilia in hBEpiCs, in evidence of significant upregulation of FOXJ1 (a master regulator of motile cilogenesis [237]) at several orders of magnitude higher than that on PET control (p<0.0001, Figure 5.4 A1) in a short period of time (14 days). This suggests an earlier promotion of cilogenesis in hBEpiCs cultured on 3D-TIPS+Collagen scaffolds since cilogenesis typically takes around 21 to 28 days to develop [238]. The microvilli (stained by acetylated alpha tubulin, Figure 5.4 B1-F1) formed on 3D-TIPS+Collagen scaffold monoculture were longer (~7 μm in length) and thicker in shape compared to the untreated (~4 μm) and PET control (~2 μm) (Figure 5.5, and Table 5.3). The average cilia length in a human airway (healthy non-smoker individual) has been reported to be ~5 μm [239], but only ~0.5 μm on PET control and ~1 μm for the human bronchial epithelial Calu-3 cell line monocultures on hyaluronan-collagen scaffolds [225].

MUC5AC glycoprotein is a substantial component of the respiratory mucus coating, and an indicator of mucociliary epithelial cell differentiation [240, 241]. 3D-TIPS+Collagen scaffolds were able to significantly increase and maintain the expression of MUC5AC gene compared to the untreated ones (p<0.01) and the standard PET cell insert culture (p<0.05) from week 1 to week 4 (Figure 5.6 A1, A2 and B1-F1), which is normally used to induce mucus secretion from epithelial cells. This indicates the effect of the ECM components on the epithelial cell response which may be contributed by the presence of collagen network within the hybrid scaffolds [242]. A similar trend of higher secretion of the
glycoprotein through MUC5AC mRNA translation on 3D-TIPS+Collagen scaffolds at day 21 seen by immunofluorescence and ELISA analysis (Figure 5.6 B1-F1, and Figure 5.6 A2) manifested more goblet cells differentiation. The respiratory mucus secreted by goblet cells is an important biofunction of the pulmonary tract and has a prominent role as a defence barrier. hBEpiCs cells cultured on 3D-TIPS+Collagen scaffolds also enhanced the expression of tight junction associated cytoplasmic scaffolding protein ZO-1 (Figure 5.4 A3, and Figure 5.4 B2-F2) and adherens junction associated transmembrane protein e-cadherin (Figure 5.6 B1 to F1-1), indicating the formation of an epithelial barrier layer (Figure 5.4 B2-F2). A significant upregulation in ZO-1 gene expression (Figure 5.4 A3, p<0.05) and translated ZO-1 protein expression (Figure 5.4 A4, p<0.01) were exhibited on 3D-TIPS+Collagen scaffolds compared to PET cell culture inserts and untreated 3D-TIPS. The immunofluorescent detection of ZO-1 (Figure 5.4 B2-F2) and e-cadherin (Figure 5.6 B1 to F1-1) visualized the intercellular mesh-like network of tight junctions and adherens junctions respectively, which are characteristic of cell-cell adhesion involved in reorganization of the actin cytoskeleton in epithelial monolayers, similar to the human bronchial epithelial Calu-3 cell line [231]. The two junctions provide different functions, with adherens junctions initiating cell-cell contacts and mediating the maturation and maintenance of the contact, and tight junctions regulating the paracellular pathway for the movement of ions and solutes in between cells [243]. Their localization in the cell periphery and their affiliation with ZO-1 protein and e-cadherin are recognized as a core component of the barrier integrity [244]. In addition, detection of f-actin on the hBEpiCs circumference reinforced the hypothesis of the presence of an epithelial barrier (Figure 5.2).

The capacity of the 3D-TIPS hybrid scaffolds was substantially boosted by an epithelial fibroblast-like co-culture model: hBEpiCs co-cultured with either hBFs or hBM-MSCs outperformed the monoculture model. It is of note that the digitally printed porous architecture of the 3D-TIPS scaffolds has been demonstrated to promote cell migration and proliferation with their own deposited ECM components (in Chapter 3, and 4), as well as to guide the growth of vascular capillary network in vivo (in Chapter 6). This is further testified by the immunofluorescent and histological images in Figures 5.4-5.7, where the thin epithelial layer was closely integrated with co-cultured cells growing within interconnected pores of the scaffolds. Gene expression of the markers of mucus secretion (MUCA5AC), barrier formation (ZO-1) and ciliation (FOXJ1) in hBEpiCs and related mRNA translated protein expression were all accelerated in the co-cultured on 3D-TIPS+Collagen scaffolds, reach the highest with hBM-MSCs. The significance also differed in terms of gene expression of MUCA5AC, ZO-1, FOXJ1, and keratin 18 at week 3 in co-cultures with hBM-MSCs and hBFs (p<0.01) (Figure 5.4, and Figure 5.6). More goblet cells (marked by MUCA5C) and fully elongated cilia (marked by acetylated alpha tubulin, Figure 5.4 B1-F1) were observed in both co-culture scaffolds but more prominence in terms of cilia length and diameter was observed by NanoFab-He-ion FIB images in co-cultures with hBM-MSCs (Figure 5.5). The differentiated epithelial layer in the in vitro co-culture with hBM-MSCs on 3D-TIPS+Collagen scaffolds consisted of ~36% goblet cells, ~55% ciliated cells and ~9% non-ciliated cells , a step closer to the human airway epithelium of a healthy individual, reported with relative cell proportions of ~20% goblet cells, ~60% ciliated cells and ~20% basal cells [245, 246] (Figure 5.7). Both tight junctions and adherens junctions were well constructed to hold well stratified epithelium, in particular, adherens
junctions grew deeper, integrating tightly with the hBM-MSC supporting layer. Overall, these results demonstrated that co-culture with either hBFs or hBM-MSCs on 3D-TIPS+Collagen scaffolds have promoted more initiation and stabilization of cell adhesion, regulation of the actin cytoskeleton, intracellular signalling and transcriptional regulation during epithelium. Such enhancements could be postulated that a culmination of signalling events regulated by both the scaffold and cellular factors, especially from hBM-MSCs, could substantially increase the cell-cell contacts and adhesion, and accelerate the formation, maintenance and function of the epithelium barrier.

TEER results (Figure 5.8) provide a measure of the integrity and permeability of the epithelial layer. The increase of TEER values (Figure 5.8 A-B) and decrease of paracellular permeability (Papp) (Figure 5.8 C) on the scaffold from monoculture to co-culture corroborated the development of tight junctions and adherens junctions within the epithelial layer [247]. TEER values have been reported in the range of 300 to 650 Ωcm² for rabbit tissue and human primary epithelial cell cultures [248], but not for ex vivo human tracheal or lung tissue. Further analysis of paracellular permeability (Papp) using FD70 confirmed that the epithelial barrier formed on the hybrid collagen functionalized 3D-printed scaffold was less permeable to solute flux (Figure 5.8 C). This can be explained in terms of a predicted smaller pore radii of the cell layers [231, 249] more evident on the hybrid composite scaffold type (Figure 5.8 D). The Papp values calculated for the composite 3D printed scaffold (0.6×10⁻⁴, 0.2×10⁻⁴ and 0.1×10⁻⁴ cm/min for monoculture and co-culture conditions with HDFs and hBM-MSCs respectively) were smaller compared to previously reported data using collagen-hyaluronate scaffolds (3×10⁻⁴ cm/min) [225]. It is of note that the asymmetry in the diffusion was drastically diminished in the co-culture with hBM-MSCs, which may be correlated to a more uniform and aligned stratified epithelium layer tightly integrated with hBM-MSCs underneath via deeper adherens junctions. Overall, a physiologically relevant in vitro tracheobronchial epithelial model has been developed on the interpenetrated hybrid network of the 3D-TIPS+Collagen scaffolds.

5.5 Conclusion

This chapter described the development of hybrid composite 3D-TIPS scaffolds as a 3D in vitro airway model by indirectly 3D printing. The epithelium on the scaffolds presented is a synergetic bioengineering combination of collagen hydrogel interpenetrated within a thermoresponsive elastomeric nanohybrid network, epithelial cell culture and bi-layered co-culture with either hBFs or hBM-MSCs underneath. The 3D hybrid scaffolds demonstrated their capacity to support the growth and differentiation of human bronchial epithelium, in addition to epithelial fibroblast-like co-cultures, generating biological structures and functions close to the human respiratory tract tissue. Co-culture with hBM-MSCs accelerated the maturation of the epithelium through promotion of more cell-cell interaction and regulation of the actin cytoskeleton, intracellular signalling and transcriptional regulation. The 3D-TIPS indirectly printing approach offers a customizable reproducible technology to generate a physiologically relevant 3D biomimetic system to advance our understanding of airway disease. The biologically responsive scaffold will serve as a future platform for personalized surgical reconstruction and regeneration.
Chapter 6

Cellular Responses to Thermoresponsive Stiffness Softening Elastomer Nanohybrid Scaffolds: An In Vitro and In Vivo Study

6.1 Introduction

Clinically available synthetic scaffolds and implants are often stronger and stiffer than the surrounding tissues. This may be due to the focus of most design and manufacture processes on optimizing biomaterials’ mechanical stability, inertness and non-toxicity without consideration of how scaffolds are likely to adapt to stimuli in its implanted environment. On the other hand, early inflammation is common after implantation [250, 251], stimulating a strong foreign body reaction and fibrosis response, which results in disorganized collagen fibres and decreased tissue strength due to fibrous scar formation [252]. Healthy bone tissue often remodels in response to the stress change due to the mismatch of mechanical properties between a hard and stiff implant and the bone tissue, and becomes less dense and weaker, known as the stress-shielding effect [253, 254]. In severe cases, this causes aseptic loosening of the implant in the absence of infection and can cause device or organ failure [255]. Thus, an ideal scaffold/implant should have the ability to alter both the surrounding environment and the cellular response to enhance positive tissue remodelling, integration and regeneration in and around it. Macrophage polarization (i.e. M1 to M2 macrophage phenotype) has been shown to regulate a regenerative versus fibrotic healing phenotype [256], and it has been reported that the mechanical properties of the scaffolds can influence scar formation via effects on the organization of fibroblasts infiltrating the wound bed and the subsequent orientation of deposited extracellular matrix (ECM) [257].

Advancements in surface topography and bulk modifications have paved the way to improving tissue integration of implants and scaffolds, and their implantation need not necessarily result in encapsulation. In particular, an appropriately porous structure can be an effective approach to maintaining a scaffold’s material composition whilst reducing stiffness mismatch [258] and is essential to allow vascularization and tissue ingrowth within the scaffold. This in turn increases the degree of tissue integration with improved chances for long term fixation of the implants via biological anchorage [259]. It has been well recognized that the interface between scaffold and biological tissue determines the long-term in vivo integration of the implant [260, 261]. However, the mechanobiological factors which contribute to the
development and maintenance of a functional interface are not fully understood, largely due to biological variation and inaccessibility of the implantation site to mechanical study. Most biomaterial stiffness studies have been performed through chemical crosslinking using static \textit{in vitro} cell culture conditions, which do not directly relate to the true \textit{in vivo} dynamic environment. Little has been reported on the \textit{in vivo} tissue responses to changes in scaffold stiffness or viscoelasticity.

Here, 3D-TIPS scaffolds with different initial stiffness and hierarchical porous structures were further revealed during stiffness softening \textit{in vitro} and \textit{in vivo}. The viability of mouse embryonic dermal fibroblasts on the scaffolds in vitro was validated. Subcutaneous implantation in a rat model provided evidence that the cellular response, including growth of tissue and blood vessel networks, and provoked inflammatory response to the scaffolds with varying starting stiffness and 3D interconnected porous structures were regulated by their stiffness softening.

6.2 Materials and methods

6.2.1 Fabrication of elastomer nanohybrid scaffolds

Non-degradable poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) scaffolds were manufactured by an in-house by 3D-TIPS technique. Briefly, PUU-POSS was synthesized as needed, adapted from a previously described protocol as in Section 2.2.1 Chapter 2. PUU-POSS was coagulated at different conditions: Cryo-Coagulation (50CC), Cryo-Coagulation + Heating (50CC+H), and Room Temperature Coagulation + Heating (50RTC+H), following a previously described protocol in Section 3.2.1 and Table 3.1 Chapter 3.

6.2.2 Mechanical characterization of structure of the scaffolds

Static tensile mechanical properties of the scaffolds \((n=6\) per condition) before and after incubation at body temperature up to 28 days were tested at wet condition. Detailed descriptions were previously referred in Section 2.3 Chapter 2.

The morphology of the surface and cross-section of the dried scaffolds was examined using a field emission scanning electron microscope (Zeiss Supra 35VP FE-SEM, Germany). The phase structure was further examined via X-ray diffraction (XRD Bruker D8 Advance, Germany). General descriptions were previously referred in Section 2.4 Chapter 2.

6.2.3 \textit{In vitro} experiments

6.2.3.1 Cell proliferation and viability

Mouse embryonic dermal fibroblasts (3T3-J2 cells) proliferation and viability descriptions were previously referred in Section 2.4.14 Chapter 2
6.2.3.2 Extracellular collagen deposition

Quantification of extracellular collagen production was described in detail in Section 2.4.5 Chapter 2. The amount of extracellular acid-soluble collagen (types I-V) was measured in cells cultured on the 3D scaffolds (n=4 per group) at days 1, 3 and 7.

6.2.3.3 Immunohistochemistry by confocal microscopy

General descriptions were previously referred in Section 2.4.7 Chapter 2.

6.2.3.4 Morphology of cell-seeded scaffolds

Following three rinses with distilled water at day 7, the cell-laden scaffolds (n=2 per group) were dehydrated through a series of graded ethanol solutions and air-dried. Dried constructs were sputter-coated with gold and observed by SEM (Zeiss Supra 35VP FE-SEM, Germany) as shown in Section 2.4.8 Chapter 2.

6.2.3.5 Histological analysis of cell-seeded scaffolds

Cell-laden scaffolds (n=2 per group) were fixed in 4% PFA in saline buffer at day 7, embedded in paraffin wax and cut into 4 μm thick sections using a Leica RM2235 (Leica Microsystems Ltd., Milton Keynes, UK) microtome. Haematoxylin and eosin (H&E) staining was performed to examine gross cell location and morphology.

6.2.4 In vivo experiments

6.2.4.1 Scaffold implantation

Detailed description can be referred to Section 2.5.1 Chapter 2.

6.2.4.2 Characterization of the structure and mechanical properties of the explants

Each explant (n=6 per group) underwent tensile mechanical testing analysis using an Instron 5655 tester (Instron Ltd., Norwood, MA, USA). The phase structure of the explants (n=2 per group) was examined via X-ray diffraction (XRD Bruker D8 Advance, Germany). Detailed description can be referred to Section 2.3 Chapter 2.

6.2.4.3 Histology and immunohistochemistry (IHC) assessments

Detailed description can be referred to Section 2.5.2 Chapter 2.

6.2.5 Data analysis

All quantitative data was presented as standard deviation (SD) of the mean values. Statistical analysis of the results was performed using Graph-Pad Prism 6 (GraphPad Software San Diego, USA). For
comparisons across more than two groups, statistical significance was calculated by two-way analysis of variance (ANOVA), with Tukey multiple comparison post-hoc analysis where a value of \( p < 0.05 \) was considered statistically significant.

6.3 Results

6.3.1 In vitro results

PUU-POSS scaffolds were seeded with embryonic mouse 3T3-J2 fibroblasts to investigate the in vitro cellular response to the scaffolds prior to implantation. Cells exhibited greater metabolic activity and proliferation on 50CC scaffolds, with the highest initial tensile modulus (in Chapter 3 Table 3.7) and the most hierarchical porous structure (in Chapter 3 Table 3.7), compared to the rest of the groups \( (p < 0.01) \), as seen by alamarBlue® and total DNA assays over the course of 14 days (Figure 6.1 A-B). The content of extracellular collagen per cell (Figure 6.1 C) also remained significantly \( (p < 0.01) \) higher on the 50CC group at all day points, followed by the 50CC+H sample. Furthermore, confocal microscopy at day 7 confirmed greater cellular activity and organization in 50CC scaffolds as seen by immunofluorescent staining and 3D reconstructions of fluorescent intensity (Figure 6.1 D-F). SEM images at day 7 (Figure 6.1 D4-F4) show well-spread morphologies of typical fibroblasts attached on the scaffold surface, and histological images by H&E staining of the cross section of the scaffolds at day 7 (Figure 6.1 D5-F5) indicate good integration of the cells within the porous network, most prominently in the 50CC scaffold. By day 14, both cell metabolic activity and total DNA decreased after reaching confluence.
Figure 6.1 *In vitro* cellular response of Mouse 3T3-J2 cells to PUU-POSS scaffolds by 3D-TIPS with different thermal processing conditions. (A) alamarBlue® fluorescence assay, (B) total DNA analysis, and (C) extracellular acid-soluble collagen (types I-V) deposition. Confocal microscopy images (×10 and
×20 objective lens) at day 7 with cells stained for f-actin (green) and counterstained nuclei (red) for (D1-D2) 50CC, (E1-E2) 50CC+H and (F1-F2) 50RTC+H. (D3-F3) 3D reconstructions of fluorescence light intensity by confocal microscopy at day 7. (D4-F4) SEM images of cell attachment and morphology at day 7. (D5-F5) Histological images of the cross sections of the scaffold at day 7 by H&E staining. **p<0.01; ***p<0.001; ****p<0.0001; errors bar in SD.

6.3.2 In vivo results

6.3.2.1 Effect of the scaffolds on cellular infiltration and matrix deposition during in vivo implantation

The scaffolds were subcutaneously implanted under the rat back skin (Figure 6.2 A-C) for up to 12 weeks. The static tensile elastic modulus, tensile strength, strain at break and toughness of explanted scaffolds (Figure 6.2 D-G, and Tables 6.2-6.4) calculated from stress-strain curves (Figure 6.2 H-J) were shown proportional increases at all three-time points tested due to reinforcement by tissue ingrowth (Figure 6.2 A-C). The 50CC scaffolds exhibited the highest mechanical properties at all time points, but non-significant differences were found between the groups at week 12 after their stiffness relaxation. The crystalline structure of the explants was evaluated at weeks 4, 8 and 12 with XRD (Figure 6.2 K-M, and Table 6.5). Before implantation, the CC scaffold presented two sharp Bragg diffraction peaks at 2θ=20 ° and 23.4º, and one broader halo peak at around 19.9º, with inter-planar spacing (d-spacing) of 0.44 nm and 0.38 nm, as the lateral distance in the interfaces of crystallized soft segments. For the CC+H group, the ordered crystal lattice structure almost disappeared, relaxing the long-distance order to a quasi-random amorphous structure with a similar diffraction profile to that of the RTC+H group (comprising three broad halo peaks, including a broadening halo peak at 2θ =19.9° and a shoulder apparent at an approximately lower angle of 2θ =12.0°). After 3 months of in vivo implantation, all scaffolds exhibited similar XRD spectrum after stiffness relaxation, where the more pronounced spectra halo peaks from all explants echoed the unique thermodynamically stable nanophase structure of the nanohybrid’s rubber phase, in agreement with the results in vitro in Chapter 3 Figure 3.6
Figure 6.2 Physico-mechanical characterization of PUU-POSS explants by 3D-TIPS with different thermal process conditions (A-C) explanted scaffolds (50% infill density) after implantation: (A) 50CC, (B) 50CC+H, and (C) 50RTC+H. (D-J) Mechanical characterization of the scaffolds before and after implantation for weeks 4, 8 and 12: (D) tensile modulus (at 50% strain), (E) ultimate tensile strength (breaking point), (F) strain at break, (G) toughness, and (H-J) stress-strain curves; (K-M) XRD spectra of the explants before and after implantation for weeks 4, 8 and 12. ****p<0.0001, errors bar in SD.
Table 6.1 Tensile modulus at 50% strain of the scaffold explants at weeks 4, 8 and 12.

<table>
<thead>
<tr>
<th>Tensile modulus (MPa)</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>1.1 (±0.1)</td>
<td>0.8 (±0.1)</td>
<td>0.4 (±0.1)</td>
</tr>
<tr>
<td>Week 4</td>
<td>2.5 (±0.4)</td>
<td>2.1 (±1.4)</td>
<td>1.6 (±0.2)</td>
</tr>
<tr>
<td>Week 8</td>
<td>4.0 (±0.6)</td>
<td>3.7 (±0.8)</td>
<td>3.1 (±0.9)</td>
</tr>
<tr>
<td>Week 12</td>
<td>7.0 (±1.5)</td>
<td>6.1 (±1.4)</td>
<td>5.9 (±1.5)</td>
</tr>
</tbody>
</table>

Table 6.2 Strain at break of the scaffold explants at weeks 4, 8 and 12.

<table>
<thead>
<tr>
<th>Strain at break (%)</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>179 (±18)</td>
<td>186 (±19)</td>
<td>146 (±15)</td>
</tr>
<tr>
<td>Week 4</td>
<td>340 (±24)</td>
<td>310 (±61)</td>
<td>291 (±71)</td>
</tr>
<tr>
<td>Week 8</td>
<td>444 (±73)</td>
<td>423 (±72)</td>
<td>406 (±122)</td>
</tr>
<tr>
<td>Week 12</td>
<td>522 (±70)</td>
<td>494 (±66)</td>
<td>454 (±80)</td>
</tr>
</tbody>
</table>

Table 6.3 Ultimate tensile strength (breaking point) of the scaffold explants at weeks 4, 8 and 12.

<table>
<thead>
<tr>
<th>Ultimate tensile strength (MPa)</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>1.6 (±0.1)</td>
<td>0.9 (±0.1)</td>
<td>0.7 (±0.1)</td>
</tr>
<tr>
<td>Week 4</td>
<td>1.1 (±0.4)</td>
<td>1.0 (±0.5)</td>
<td>0.8 (±0.2)</td>
</tr>
<tr>
<td>Week 8</td>
<td>1.9 (±0.4)</td>
<td>1.9 (±0.5)</td>
<td>1.2 (±0.4)</td>
</tr>
<tr>
<td>Week 12</td>
<td>2.8 (±0.5)</td>
<td>2.6 (±0.8)</td>
<td>2.4 (±0.3)</td>
</tr>
</tbody>
</table>

Table 6.4 Toughness of the scaffold explants at weeks 4, 8 and 12.

<table>
<thead>
<tr>
<th>Toughness (J.m(^{-3})10(^4))</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>137 (±12)</td>
<td>145 (±11)</td>
<td>112 (±17)</td>
</tr>
<tr>
<td>Week 4</td>
<td>412 (±24)</td>
<td>369 (±66)</td>
<td>350 (±79)</td>
</tr>
<tr>
<td>Week 8</td>
<td>523 (±72)</td>
<td>462 (±81)</td>
<td>405 (±161)</td>
</tr>
<tr>
<td>Week 12</td>
<td>599 (±99)</td>
<td>523 (±77)</td>
<td>443 (±89)</td>
</tr>
</tbody>
</table>
Table 6.5 Analysis of WAXD spectra of the explants during implantation. Degree of crystallinity (Dc, %), d-spacing (d, Å) of semicrystalline structure and broad halo peaks of amorphous structures.

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>W0</th>
<th>W4</th>
<th>W8</th>
<th>W12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2θ</td>
<td>d</td>
<td>Dc</td>
<td>2θ</td>
</tr>
<tr>
<td>50CC</td>
<td>Sharp peak 1</td>
<td>20.0</td>
<td>4.4</td>
<td>37.6</td>
</tr>
<tr>
<td></td>
<td>Sharp peak 2</td>
<td>23.2</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 3</td>
<td>30.0</td>
<td>30.6</td>
<td>31.3</td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 4</td>
<td>40.5</td>
<td>41.6</td>
<td>41.9</td>
</tr>
<tr>
<td>50CC+H</td>
<td>Sharp peak 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sharp peak 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 2</td>
<td>19.2</td>
<td>19.2</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 3</td>
<td>30.1</td>
<td>28.8</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 4</td>
<td>41.3</td>
<td>42.2</td>
<td>42.3</td>
</tr>
<tr>
<td>50RTC+H</td>
<td>Sharp peak 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sharp peak 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 2</td>
<td>19.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 3</td>
<td>26.0</td>
<td>25.9</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td>Broad halo peak 4</td>
<td>42.3</td>
<td>41.9</td>
<td>42.6</td>
</tr>
</tbody>
</table>

H&E staining and MT of subcutaneously implanted scaffolds revealed good ingrowth of tissue in all scaffold types throughout their interconnected porous networks (Figure 6.3 A-L, and Figure 6.4-6.6). The thickness of the aligned tissue ingrowth within the various scaffolds was quantified after implantation at weeks 4, 8 and 12 (Figure 6.3 M, and Figure 6.4). Faster and greater amount of aligned ingrowth tissue was reported on the 50CC scaffold compared to the rest of the groups (p<0.001).

A large area of H&E stained histological sections at low magnification is shown in Figure 6.4, indicating the typical patterns of tissue ingrowth, which is further analysed in Figure 6.7. Internal hydrostatic pressure was applied to the scaffolds once implanted, due to bending confinement under the rat back skin, with a combination of compression and tension stresses distributed within them as illustrated in (Figure 6.7 A-B). This is reflected by the obvious deformation of the polymer macrostructure in histological samples in combination with matrix deposition and tissue infiltration (Figure 6.3-6.6). Consequently, tissue ingrown following the digitally printed geometry of the interconnected tunnels (left by dissolution of the printed PVA network) in response to their local microenvironment. Figure 6.7 C-N shows some typical H&E stained structure of the ingrown tissue in response to the geometry of the macro- to micro-porous structure and possible local stresses distributed. At the vertical pore junctions
of the tunnels (i.e. cross junction of printed PVA struts), new tissue was round in shape with concentric circularly aligned microfilament bundles (i.e. elongated myofibroblast and collagen fibres), whereas most microvascular vessels grew perpendicularly through the less aligned central tissue (Figure 6.7 C-E). Despite printing symmetric orthogonal patterns, the short and long dumbbell-shaped ingrown tissues between two junctions appeared and showed distinctly different orientations of myofibroblasts and collagen fibres, with either perpendicular (Figure 6.7 F) or parallel alignment with respect to the tunnels (Figure 6.7 I and L). Such different confinement may be induced by the local stress conditions of the scaffold, where tensile stress stretched the struts while compression stress shortened the distance of the channels. In addition, the long dumbbell tissue grew relatively slower compared with the concentric areas at the earlier (4-week) time point, perhaps due to a less efficient transport of nutrients through the elongated tunnel horizontally (Figure 6.7 E, H, K and N). At 12 weeks, the minimum diameter ($D_{\text{min}}$) of ingrown tissue was the largest in the 50CC scaffold group and smallest in the 50CC+H group, consistent with the original micro/macropore diameters of the scaffolds in Chapter 3 Table 3.4.
**Figure 6.3** Cellular infiltration and matrix deposition in PUU-POSS scaffolds produced after subcutaneous implantation for a week 12: (A) tissue integration of middle-in-plane by Hematoxylin and Eosin (H&E) staining in 50CC; (B) collagen production by Masson’s trichome (MT) staining in 50CC; (C) endothelial cell infiltration in 50CC as identified by CD31 staining, which is used as a marker of angiogenesis, and (D-F) enlarged views respectively. (G-I) Middle cross-sectional view and (J-L) enlarged view of the 50CC scaffolds, respectively for H&E, MT and endothelial cell staining. (M-N) Quantification of cellular integration and growth by 4 and 12 weeks of the various scaffolds (M) thickness of aligned tissue ingrowth; (N) capillary infiltration density of ingrowth tissue. **p<0.01, ****p<0.0001, errors bar in SD; (n=10, four scaffolds in each group at each time point).
Figure 6.4 Hematoxylin & Eosin (H&E) stained histological structure of middle in-plane of 50CC scaffold explants at week 12 depicting tissue ingrowth within the scaffold network, ×2 magnifications.
**Figure 6.5** Subcutaneous implantation of 50CC+H scaffolds at week 12: (A) tissue integration of middle-in-plane of the 50CC+H scaffold by Hemotoxylin and Eosin (H&E) staining; (B) collagen production by Masson’s trichome staining; (C) endothelial cell infiltration as identified by CD31 staining, which is used as a marker of angiogenesis, and (D-F) enlarged views respectively. (G-I) Middle cross section view and (J-L) enlarged view.

**Figure 6.6** Subcutaneous implantation of 50RTC+H scaffolds at week 12: (A) tissue integration of middle-in-plane of the 50RTC+H scaffold by Hemotoxylin and Eosin (H&E) staining; (B) collagen production by Masson’s trichome staining; (C) endothelial cell infiltration as identified by CD31 staining, which is used as a marker of angiogenesis, and (D-F) enlarged views sections respectively. (G-I) Middle cross-sectional view and (J-L) enlarged middle cross-sectional view.
Figure 6.7 Tissue ingrowth within the network of PUU-POSS scaffolds by 3D-TIPS with different thermal processing conditions at weeks 4 and 12 in vivo: (A) bend loading condition of the implanted scaffold due to hydrostatic pressure under the rat skin; (B) stress distribution of compression and tension across the scaffold cross-section under bending load; (C-N) H&E histological structure and schematic diagrams of stress condition and statistical analysis of the ingrowth of tissue; (C, D, E) concentric aligned tissue at the junction of the scaffold; (F, G, H) short dumbbell tissue with the compressed channels; (I, J, K) long dumbbell tissue between the elongated channel; (L, M, N) aligned tissue in long tunnels. *p<0.05; ***p<0.001, ****p<0.0001, errors bar in SD (n=10 in each group at each time point).
6.3.3.2 Effect of stiffness memory on vascularization in vivo

Figure 6.8 Angiogenesis in PUU-POSS scaffolds by 3D-TIPS with different thermal processing conditions. (A-L) 3D image reconstruction of immunofluorescent staining of anti-CD31 marker for blood capillaries at weeks 4 and 12 for the various scaffolds. (M) Mean volume fraction of blood capillaries of total tissue/scaffold volume at weeks 4, 8 and 12. ****p<0.0001, errors bar in SD (n=10, four scaffolds in each group at each time point).
Ingrowth of blood capillaries were clearly visualized within the implanted scaffolds as early as 4 weeks post implantation and continued to increase until 12 weeks after implantation, as demonstrated by anti-CD31 immunofluorescence (Figure 6.8 A-L). The capillary volume fraction (CVF), i.e. the volume of blood capillaries occupied within the overall volume of the scaffold, was used to compare the functionality of the angiogenic response of the host towards the 3D scaffolds in each group (Figure 6.8 M). The CVF increased from week 4 towards week 12 for each scaffold group. Higher CVF values were observed in the 50CC group compared with the rest of the scaffolds (p<0.0001) at all time points. This is consistent with a greater capillary infiltration density of ingrowth tissue for the 50CC group (Figure 6.3 C, N). The 50RTC+H group exhibited the smallest CVF (Table 6.6).

Table 6.6 Proportion of total tissue/scaffold volume occupied by blood capillaries at weeks 4, 8 and 12.

<table>
<thead>
<tr>
<th>Capillary (%)</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>10.9 (±1.3)</td>
<td>5.8 (±2.2)</td>
<td>3.1 (±1.9)</td>
</tr>
<tr>
<td>Week 8</td>
<td>24.7 (±3.5)</td>
<td>12.2 (±4.2)</td>
<td>8.0 (±3.8)</td>
</tr>
<tr>
<td>Week 12</td>
<td>30.1 (±4.2)</td>
<td>19.9 (±5.2)</td>
<td>14.3 (±5.3)</td>
</tr>
</tbody>
</table>

6.3.2.3 Effect of stiffness softening on the macrophage and T-cell proliferative responses in vivo

The effect of the scaffolds towards macrophage activation and polarization was studied by immunohistochemistry with markers against CD68+ and CD86+ (M1 pan-macrophage/monocyte marker and macrophage marker), and CD163+ cell subsets (M2 phenotype). Macrophages are plastic cells and the M1/M2 phenotype is widely used to distinguish between different macrophage activation states. The M1 macrophage phenotype (classically activated macrophage) is known to induce prototypic inflammatory responses; in contrast, cells of the M2 phenotype (alternatively activated macrophages) are able to antagonize prototypic inflammatory responses. All implanted scaffolds in vivo were able to modulate the inflammatory reaction by driving the macrophage response (Figure 6.9). In particular, there was a decrease in the density of CD68+ and CD86+ cells in the surrounding tissue with increasing time periods (Figures 6.10 and 6.12 towards week 12 (Figures 6.11, and 6.13) (p<0.01 CD68+ and p<0.001 CD86+). Conversely, an increase in the density of CD163+ cells (M2 phenotype) (Figure 6.20 C, and Table 6.9) was observed at week 12 (Figure 6.15) compared to week 4 (Figure 6.14). By computing the macrophage polarization ratio M1/M2 (i.e. Figure 6.20 D-E in terms of CD68+/CD163+ and CD86+/CD163+ respectively, and Tables 6.10-6.11), which determines the inflammatory vs. reparative potential during implantation of the scaffold, it was significantly lower for both the 50CC and 50CC+H groups (p-value non-significant) compared to the 50RTC+H samples (p<0.0001) for all time points.

The T-cell proliferative response of the scaffolds after implantation was also studied by immunohistochemistry with markers against cell subsets CD3+ and CD4+. The corresponding numerical
density histogram (Figure 6.20 F-G, and Tables 6.12 - 6.13) indicates a decrease in the CD3+ and CD4+ T-cell proliferative response within all scaffold groups from week 4 (Figures 6.16, and 6.18) to week 12 (Figures 6.17, and 6.19). The majority of the CD3+ hyporesponse proliferation shown by the scaffolds is therefore due to a decrease in the CD4+ proliferative response associated directly or indirectly with the presence of M2 monocytes. The macrophage polarization and abundance data indicate that both the 50CC and 50CC+H scaffolds, with a greater thermoresponsive stiffness softening mechanism compared to the 50RTC+H samples, polarized infiltrating macrophages towards a regenerative phenotype, consistent with the matrix deposition and cellular infiltration patterns seen in these scaffold types.
**Figure 6.9** Immunohistochemistry of the host macrophage response in PUU-POSS scaffolds by 3D-TIPS with different thermal processing conditions. Week 12, tissue integration of middle-in-plane view of the scaffolds by CD68/CD86 (M1 marker), CD163 (M2 marker) and CD3/CD4 (T lymphocyte markers) staining. Scale bars: 100 μm.

**Figure 6.10** Immunohistochemistry of the host macrophage response towards scaffolds *in vivo* at week 4. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD68 (M1 pan-macrophage/monocyte marker) staining at (A-C, G-I) ×4 and (D-F, J-L) ×20 magnifications.
Figure 6.11 Immunohistochemistry of the host macrophage response towards scaffolds in vivo at week 12. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD68 (pan-macrophage/monocyte marker) staining at (A-C, G-I) ×4 and (D-F, J-L) ×20 magnifications. (M) Negative control (rat appendix); (N) positive control (rat liver). Scale bars: 100 μm
Figure 6.12 Immunohistochemistry of the host macrophage response towards scaffolds in vivo at week 4. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD86 (M1 macrophage marker) staining at (A-C, G-I) ×4 and (D-F, J-L) ×20 magnifications.
Figure 6.13 Immunohistochemistry of the host macrophage response towards scaffolds in vivo at week 12. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD86 (M1 macrophage marker) staining at (A–C, G–I) ×4 and (D–F, J–L) ×20 magnifications. (M) Negative control (rat appendix); (N) positive control (rat liver). Scale bars: 100 μm
Figure 6.14 Immunohistochemistry of the host macrophage response towards scaffolds *in vivo* at week 4. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD163 (M2 macrophage marker) staining at (A-C, G-I) ×4 and (D-F, J-L) ×20 magnifications.
Figure 6.15 Immunohistochemistry of the host macrophage response towards scaffolds in vivo at weeks 12. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD163 (M2 macrophage marker) staining at (A-C, G-I) x4 and (D-F, J-L) x20 magnifications. (M) Negative control (rat appendix); (N) positive control (rat liver). Scale bars: 100 μm
Figure 6.16 Immunohistochemistry of the host T lymphocyte response towards scaffolds *in vivo* at week 4. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD3 (T lymphocyte marker) staining at (A-C, G-L) ×4 and (D-F, J-L) ×40 magnifications.
Figure 6.17 Immunohistochemistry of the host T lymphocyte response towards scaffolds in vivo at week 12. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD3 (T lymphocyte marker) staining at (A-C, G-I) ×4 and (D-F, J-L) ×20 magnifications. (M) Negative control (rat appendix); (N) positive control (rat spleen). Scale bars: 100 μm
Figure 6.18 Immunohistochemistry of the host T lymphocyte response towards scaffolds in vivo at week 4. Tissue integration of middle-in-plane (A-F) and cross-sectional view (G-L) of the scaffolds by CD4 (T lymphocyte marker) staining at (A-C, G-I) ×4 and (D-F, J-L) ×20 magnifications.
Figure 6.19 Immunohistochemistry of the host T lymphocyte response towards scaffolds in vivo at week 12. Tissue integration of middle-in-plane (A-F) and cross-sectional view (g-l) of the scaffolds by CD4 (T lymphocyte marker) staining at (A-C, G-I) ×4 and (D-F, J-L) ×20 magnifications. (M) Negative control (rat appendix); (N) positive control (rat spleen). Scale bars: 100 μm
Figure 6.20 Quantification of macrophage and T-cell response of PUU-POSS scaffolds by 3D-TIPS with different thermal processing conditions. Numerical density, as shown in the histogram, represents the number of cells across the scaffold per unit volume at weeks 4, 8 and 12; n=20 frames, 12 scaffolds in each group at each time point: (A) M1 marker CD68+; (B) M1 marker CD86+, (C) M2 marker CD163+; (D) macrophage polarization CD68+/CD163+; (E) macrophage polarization CD86+/CD163+; (F) T lymphocyte marker CD3+; (G) T lymphocyte marker CD4+.*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Table 6.7 Host pan-macrophage/monocyte response (CD68+ marker) towards the implanted scaffolds in terms of numerical density (Nv), representing the number of cells across the scaffold per unit square (Nv/mm²) at week 4, 8 and 12 (n=20 frames, 12 scaffolds in each group at each time point).

<table>
<thead>
<tr>
<th>CD68+</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>353 (±54)</td>
<td>301 (±56)</td>
<td>210 (±46)</td>
</tr>
<tr>
<td>Week 8</td>
<td>322 (±48)</td>
<td>260 (±39)</td>
<td>164 (±48)</td>
</tr>
<tr>
<td>Week 12</td>
<td>228 (±39)</td>
<td>201 (±43)</td>
<td>115 (±52)</td>
</tr>
</tbody>
</table>

Table 6.8 Host macrophage response (CD86+ marker) towards the implanted scaffolds in terms of numerical density (Nv), representing the number of cells across the scaffold per unit square (Nv/mm²) at week 4, 8 and 12 (n=20 frames, 12 scaffolds in each group at each time point).

<table>
<thead>
<tr>
<th>CD86+</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>397 (±56)</td>
<td>289 (±47)</td>
<td>152 (±39)</td>
</tr>
<tr>
<td>Week 8</td>
<td>312 (±55)</td>
<td>224 (±51)</td>
<td>132 (±45)</td>
</tr>
<tr>
<td>Week 12</td>
<td>271 (±41)</td>
<td>186 (±55)</td>
<td>96 (±53)</td>
</tr>
</tbody>
</table>

Table 6.9 Host macrophage response (CD163+ marker) towards the implanted scaffolds in terms of numerical density (Nv), representing the number of cells across the scaffold per unit square (Nv/mm²) at week 4, 8 and 12 (n=20 frames, 12 scaffolds in each group at each time point).

<table>
<thead>
<tr>
<th>CD163+</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>360 (±64)</td>
<td>294 (±65)</td>
<td>78 (±36)</td>
</tr>
<tr>
<td>Week 8</td>
<td>531 (±88)</td>
<td>434 (±76)</td>
<td>103 (±67)</td>
</tr>
<tr>
<td>Week 12</td>
<td>679 (±94)</td>
<td>534 (±78)</td>
<td>167 (±46)</td>
</tr>
</tbody>
</table>

Table 6.10 Ratio of CD68+/ CD163+ of the various scaffold groups at weeks 4, 8 and 12.

<table>
<thead>
<tr>
<th>CD68+/CD163+</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>0.9 (±0.2)</td>
<td>1.0±(0.1)</td>
<td>2.7(±0.2)</td>
</tr>
<tr>
<td>Week 8</td>
<td>0.60(±0.1)</td>
<td>0.6±(0.1)</td>
<td>1.6(±0.3)</td>
</tr>
<tr>
<td>Week 12</td>
<td>0.3(±0.04)</td>
<td>0.4(±0.1)</td>
<td>0.7(±0.1)</td>
</tr>
</tbody>
</table>

Table 6.11 Ratio of CD86+/ CD163+ of the various scaffold groups at weeks 4, 8 and 12.

<table>
<thead>
<tr>
<th>CD86+/CD163+</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>1.1(±0.3)</td>
<td>0.9(±0.1)</td>
<td>1.9(±0.3)</td>
</tr>
<tr>
<td>Week 8</td>
<td>0.6(±0.1)</td>
<td>0.5(±0.1)</td>
<td>1.2(±0.2)</td>
</tr>
<tr>
<td>Week 12</td>
<td>0.4(±0.1)</td>
<td>0.4(±0.1)</td>
<td>0.5(±0.1)</td>
</tr>
</tbody>
</table>
Table 6.12 Host T lymphocyte response (CD3+ marker) towards the implanted scaffolds in terms of numerical density (Nv), representing the number of cells across the scaffolds per unit square (Nv/mm²) at week 4, 8 and 12 (n=20 frames, 12 scaffolds in each group at each time point).

<table>
<thead>
<tr>
<th>CD3+</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>372 (±54)</td>
<td>301 (±56)</td>
<td>134 (±31)</td>
</tr>
<tr>
<td>Week 8</td>
<td>232 (±48)</td>
<td>204 (±39)</td>
<td>67 (±15)</td>
</tr>
<tr>
<td>Week 12</td>
<td>156 (±44)</td>
<td>109 (±43)</td>
<td>35 (±8)</td>
</tr>
</tbody>
</table>

Table 6.13 Host T lymphocyte response (CD4+ marker) towards the implanted scaffolds in terms of numerical density (Nv), representing the number of cells across the scaffolds per unit square (Nv/mm²) at week 4, 8 and 12 (n=20 frames, 12 scaffolds in each group at each time point).

<table>
<thead>
<tr>
<th>CD4+</th>
<th>50CC</th>
<th>50CC+H</th>
<th>50RTC+H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>301 (±61)</td>
<td>245 (±71)</td>
<td>152 (±27)</td>
</tr>
<tr>
<td>Week 8</td>
<td>252 (±42)</td>
<td>201 (±46)</td>
<td>102 (±28)</td>
</tr>
<tr>
<td>Week 12</td>
<td>122 (±32)</td>
<td>87 (±45)</td>
<td>32 (±16)</td>
</tr>
</tbody>
</table>

6.4 Discussion

*In vitro* study showed that the scaffolds promoted efficient attachment and proliferation of mouse fibroblasts within the porous structure (in Chapter 3 Table 3.7), as demonstrated by quantitative cell viability tests, morphology and histological analysis (Figure 6.1). Cells were viable on all scaffolds, with the 50CC group exhibiting significantly (p<0.01) higher cellular activity during stiffness softening, as supported by metabolic activity, total DNA and extracellular collagen deposition assays. This is reminiscent of our previously reported results using human dermal fibroblasts [262].

The digitally printed interconnected macropores and channels of the scaffolds are adequate to facilitate tissue ingrowth and accommodate microvascularization (Figures 6.2-6.7) while keeping their overall structural integrity. Despite stiffness softening of the scaffolds, the tensile mechanical properties of the explants significantly (p<0.0001) increased over time (Figure 6.2 D-G). The two main contributing factors to this were tissue ingrowth into the pores of the structures [263] and tissue remodeling via alignment of collagen fibres and elongated myofibroblasts in response to scaffolds mechanical changes (Figures 6.3-6.7). After 12 weeks implantation, non-significant differences in the tensile mechanical properties were observed between the different scaffold groups, which may be attributed to the stiffness softening effect that all the scaffolds relaxed to the same soft rubber phase. Similar to our previously reported *in vitro* study [262], cryo-3D-TIPS scaffolds (50CC) with different starting stiffness gradually relaxed through melting of the semi-crystalline structure and inverse self-assembling to a quasi-random nanophase structure (Figure 6.2 H-J) with softer hyperelasticity following implantation.
CHAPTER 6

Cellular Responses to Thermoresponsive Stiffness Softening Elastomer Nanohybrid Scaffolds: An In Vitro and In Vivo Study

The initially higher matrix stiffness of the scaffolds with their subsequent relaxation, coupled with a suitable surface pore size (in Chapter 3 Table 3.7), strongly influenced local tissue growth kinetics, corroborating in vitro data relating to cell attachment and proliferation (Figure 6.1). H&E staining and collagen deposition showed that tissue grew into various anatomical structures following the geometry of the printed interconnected macro-framework tunnels in response to the local environment (Figures 6.3-6.7), where short dumbbell tissue was seen growing in the joint tissue area, and aligned tissue grew along elongated horizontal tunnels. In particular, histological analysis demonstrated faster and greater aligned tissue ingrowth for the 50CC scaffolds. In addition to the geometry confinement, it is envisaged that these effects are due to local compression and local surface tension respectively [264, 265]. Since 50CC and 50CC+H share similar morphology and porous structure, the difference of aligned tissue ingrowth between them may be mainly attributed to the stiffness softening. Porous structure may have more influence on the difference of the tissue between 50CC+H and 50RTC+H, which is not significant (p>0.05) (Figures 6.3 and 6.7).

For a tissue to grow beyond the diffusion limit of oxygen (between 100 to 200 μm), the formation of new blood vessels is required [266]. The tissue reaction to the scaffolds included an efficient promotion of an angiogenic response, with the appearance of blood vessels as early as week 4 (Figure 6.8). The highest CVF was seen in the 50CC group, with 50RTC+H sample exhibiting the lowest CVF value. It is suggested that this greater microvascularization observed in the 50CC scaffold may be mainly promoted by a greater degree of stiffness relaxation, demonstrating the importance of the coherent scaffold-tissue stiffness matching. On the other hand, this phenomenon may also be contributed by a relatively broad hierarchy in the micro- to nano-porous structure of this scaffold group. The porous interconnectivity of scaffolds have been recognized to promote blood vessel invasion and facilitate tissue integration [267], as an appropriate macro- to micro- to nano-porosity is essential to allow nutrients to infiltrate and provide pathways for new blood vessel formation. The 50CC group, with its interconnected pores exhibited the densest capillary network generation during its stiffness relaxation period, significantly higher (p<0.0001) than that from 50CC+H group with the similar porous structure, indicating the stiffness softening may have a substantial influential role in angiogenesis (Figure 6.8 M). The lowest blood vessel count seen in the 50RTC+H scaffolds may be due to the reduced surface micro-to nano-porous structure (Table 6.1). This may reduce the surface area of the interfacial microenvironment and consequently, the diffusion of nutrients, metabolites and soluble factors throughout the scaffold. The difference of blood capillary count (Figure 6.3 N) and CVF (Figure 6.8 M) between 50CCC+H and 50RTC+H is less significant (p<0.05) maybe reflect the influence of uniformity and hierarchy of the porous structure of the scaffold on the blood vessel growth. Nevertheless, while significantly greater vascularization was observed after 3 months in all implanted scaffolds after full stiffness relaxation, further work address whether this phenomenon relates to ingrowth of existing blood capillaries within the porous structure or due to true de novo angiogenesis.

On the other hand, the highly plastic inflammatory macrophage phenotype can also profoundly influence regeneration by altering the fibrotic [268]. A reduced inflammatory response is one of the factors required for scar less wound healing and reduced fibrosis formation in implants [269], and the predominant
phenotype of resident macrophages can provide an indication of the scaffold rejection (inflammation) or acceptance following implantation and determine the stage of wound healing [270]. While M1 macrophages are known to express high levels of interleukins and pro-inflammatory cytokines that promote inflammation, M2 macrophages express low levels of these and are able to facilitate and promote tissue repair [271]. It has been shown that the mechanical and topological properties of the scaffolds can regulate macrophage responses [256, 272, 273]. Macrophages have also been demonstrated to sense their underlying substrate stiffness: higher macrophage cell spreading and attachment is seen on stiffer substrates, leading to a more severe foreign body reaction, while softer substrates promote M2-like macrophage activation towards a wound healing phenotype [274, 275].

The stiffness softening effect of the scaffolds on macrophage polarization was therefore investigated. Despite the difference between each scaffold type, the overall trend of the inflammatory response is similar with a decrease of M1 macrophages and T-cells, and an increase of M2 macrophages from week 4 to week 12 implantation (Figure 6.20). In particular, macrophage polarization from an M1 towards an M2 phenotype was observed within all implanted scaffolds, as evidenced by the reduction of CD68+ and CD86+ cells from week 4 towards week 12 (Figure 6.20 A-B) and the increase of CD163+ (Figure 6.20 C) in the scaffolds. The initial high stiffness and subsequent stiffness softening of the 50CC scaffold appeared to trigger more M1 and M2 macrophages as well as T cells from the early stage of implantation, compared to 50CC+H and 50RTC+H. In addition, the M1/M2 ratio was found to be lower for both the 50CC and 50CC+H groups than 50RTC+H scaffolds at all time points (Figure 6.20 D-E). After 12 weeks of implantation, the difference between 50CC and 50RTC+H is less significant after the long stiffness softening, indicating that the morphology and porous structure of the scaffolds also have a strong influence on the inflammatory response. It is here suggested that the stiffness relaxation effect and hierarchical porous structure exhibited by the 50CC and 50CC+H scaffolds plays a coherent role in local inflammatory response modulation and could be used as a significant parameter to aid macrophage M1 to M2 polarization. The findings of macrophage polarization are also supported by an attenuated in vivo proliferation of CD3+ and CD4+ T-cell subsets at 12 weeks (Figure 6.20 F-G) compared to week 4. Prolonged in vivo implantation periods should be explored, as should detection and quantification of inhibitory and pro-inflammatory cytokine levels. In addition, quantitative PCR of Wnt-related genes could be studied, as the Wnt signaling pathway is known to be a key mechanotransduction pathway in fibroblast regulation of wound healing [276].

6.5 Conclusion

The digitally programmed shape and interconnected macro/micro-interconnected porous structure of the thermoresponsive elastomeric PUU-POSS scaffolds by 3D-TIPS have been shown to guide and promote interfaces for tissue ingrowth and the formation of functional microvascular networks. In concordance with in vitro study, the stiffness softening, induced by physical phase transition and self-assembly of soft and hard chain segments of PUU chains, has been found to promote in vitro and in vivo cell adhesion, and proliferation, tissue ingrowth and vascularization, with no changes in molecular structure of the scaffold.
This stiffness softening effect together with the hierarchical porous structure were seen to modulate tissue ingrowth in several ways and to reduce \textit{in vivo} inflammation in a rat model for up to 12 weeks, with enhanced polarization towards the macrophage M2 phenotype. The observations indicate that the stiffness softening demonstrated by the 3D-TIPS PUU-POSS scaffolds could prove an effective route to regulate a host regenerative vs. scarring phenotype, while matching the mechanical properties of the surrounding soft tissue and improving tissue integration and healing after implantation.
Chapter 7

Conclusions and Future Perspectives

7.1 Conclusions

Tracheal tissue engineering (TTE) appears as a promising alternative to tackle trachea-related, especially long-segment tracheal defects, though challenges regarding epithelialization and re-vascularization of tracheal scaffolds still persist on the way towards clinic practice. Given these two primary challenges, the main theme of this thesis is to develop elastomeric nanohybrid scaffolds with characteristics of hierarchical porous structure, matched mechanical properties and biocompatibility using 3D printing technique for \textit{in vitro} and \textit{in vivo} investigations, aiming at potential patient-specific trachea tissue reconstructions.

A family of thermoresponsive non-degradable poly(urea-urethane)-polyhedral oligomeric silsequioxane (PUU-POSS) nanohybrid scaffolds were developed using 3D printing guided thermally induced phase transition (3D-TIPS). A wide hierarchy of porous structures and tuneable stiffness of the scaffolds were achieved by dual control of 3D printing infill density and phase separation of polymer solution at different temperatures. For the first time, an engineered trachea l model that exhibits stiffness relaxation has been reported. The stiffness-to-relaxation mechanism is a result of both isothermal viscoelasticity and thermal dynamic mechanical behaviors, as well as the order-to-quasi-random nanophase structure of PUU-POSS nanohybrid elastomer. These scaffolds with different stiffness and stiffness softening were achieved through microphase separation of PUU chains and crystallization of soft segments.

Systematic studies were carried out to prove the different roles of hierarchical micro/nano-porous structure, initial stiffness and stiffness softening in modulating cell growth and differentiation. Initial stiffness and subsequent stiffness softening of these scaffolds at body temperature were found to promote cellular proliferation of both human dermal fibroblast (HDFs) and human bone-marrow derived mesenchymal stem cells (hBM-MSCs) and regulate their differentiation as well towards the chondrogenic and osteogenic lineages. hBM-MSCs showed enhanced chondrogenic differentiation on softer scaffolds and osteogenic differentiation on stiffer ones, with similar relative expression to that of human femoral head tissue. On the other hand, the hierarchical porous structure and resulting large surface area of the scaffold were believed to enhance cellular attachment and nutrient/gas exchange flow during cell culture.
The 3D-TIPS scaffold is a suitable choice as an *in vitro* co-culture tracheobronchial epithelium model with respect to epithelialization. In addition, it could serve as a future platform model for surgical reconstruction and tracheal regeneration. The scaffold discussed a bioengineering synergy of combining a collagen-based hydrogel impregnated with 3D-TIPS scaffolds for epithelial cell culture. The multi-layered co-culture 3D scaffolds consisting of a top monolayer of differentiated epithelium, with either hBFs or hBM-MSCs proliferating within the hybrid scaffold underneath, created a tissue analogue of the upper respiratory tract, which validated these 3D printing guided scaffolds as a platform to support co-culture and cellular organization. In particular, hBM-MSCs in the co-culture system promoted an overall matured physiological tissue analogue of the respiratory system, a promising synthetic tissue for tracheal repair and reconstruction.

This thesis, for the first time, also reports cellular responses to 3D-TIPS scaffolds with different stiffness softening both *in vitro* and *in vivo* in rat models. The 3D-TIPS interconnected porous scaffolds have been shown to modulate tissue ingrowth and promote good vascularization via defined macro-pores and the micro- and nano-hierarchical structure when implanted under rats’ back skin for up to 3 months. All scaffolds exhibited polarization of the macrophage response from a macrophage phenotype type I (M1) towards a macrophage phenotype type II (M2) and down-regulation of the T-cell proliferative response with increasing implantation time; however, scaffolds with a more pronounced thermoresponsive stiffness softening mechanism exerted higher inflammo-informed effects. These results pave the way for personalized and biologically responsive soft tissue implants and implantable device with better mechanical matches, angiogenesis and tissue integration.

### 7.2 Future perspectives

As one of the outcomes of this research is for improved epithelialisation of the tissue engineered trachea, the next step for this research could be geared towards developing a 3D printed joint cartilage-epithelial analogue as a 3D *in vitro* airway model, as it has been shown that tracheal cartilage has the ability to signal to the adjacent epithelium to induce proper epithelial cell differentiation [277, 278]. The scaffold would consist of a cartilage layer formed from chondrogenic-differentiated hBM-MSCs and would be used as a primary substrate that can be co-cultured with a human epithelial cell line seeded on top. Assuming similar co-culture conditions are applied, the joint tissue analogue should demonstrate the ability to enhance the differentiation and development of human bronchial epithelium compared to non-differentiated hBM-MSCs in similar co-culture conditions.

This thesis focuses on development of 3D-TIPS processing technique to produce a representative anatomically shaped tracheal scaffold model, and investigations *in vitro* and *in vivo* biocompatibility of the scaffolds, without delicate design of trachea scaffold involved. So far it is possible to construct a 3D porcine tracheal model by using the Materialise Mimic software (MIMICS 15.0, Materialise NV, Leuven, Belgium), more accurate constructs at the scale and in the shape of the animal's trachea need to be further designed. The optimized model can then be used to print a more biomimetic trachea that can be used for *in vivo* porcine studies. More specific *in vivo* studies on the effect of stiffness softening in human cartilage and bone models also deserve further investigation. Prior to this step, the focus
would be on the evaluation of porcine in vivo variables, including the porcine of tissue integration, neo-vascular formation, tissue fibrosis/stenosis or extrusion porcine of the implants. Pre-in vivo studies are also required to improve optimization of the physicochemical structure of the polymer itself.

Furthermore, alternative polymeric materials with thermoresponsive mechanical properties (stiffness softening scaffolds) that change viscoelastic properties at temperatures closer to human body are desirable for scaffold fabrication. Integrating a stiffness softening effect and a 3D printing approach using the 3D-TIPS technique can provide a valuable combinatorial tool for improved cell growth and differentiation, making these scaffolds worthy of further consideration for future regenerative medicine and implantable devices. For example, there is a clinical need for bronchial patches and replacements in the context of broncho-pleural fistulas and lung-sparing surgery for lung cancer. Further research may be carried out regarding such thin walled tubular constructs in a wide range of diameters.
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Appendix. A

A. Thermodynamic of polymer solution and phase separation

The Onsager reciprocal relations (ORR) provide a general connection between phenomenological transport coefficients in the linear response regime in irreversible processes, such as a phase separation [48].

The basic thermodynamic potential is internal energy. In a simple fluid system at fixed volume, the internal energy density, $u$, is written:

$$du = Tds + \mu d\rho \quad \text{(E-1)}$$

where $s$ is the entropy density, $\rho$ is the mass density.

The conservation of mass is expressed locally by the fact that the flow of mass density $\rho$ satisfies the continuity equation:

$$\frac{\partial \rho}{\partial t} + \nabla \cdot J_{\rho} = 0 \quad \text{(E-2)}$$

where $J_{\rho}$ is the mass flux vector.

In the absence of matter flows, the above equation may be solved for the entropy density:

$$ds = \frac{1}{T}du - \frac{\mu}{T}d\rho \quad \text{(E-3)}$$

$T$ is the absolute temperature.

In the absence of heat flow, Fick's law of diffusion is usually written:

$$J_{\rho} = -D(\varphi/\chi) \quad \text{(E-4)}$$

where, $J_{\rho}$ is the diffusion flux vector that measures the amount of substance that will flow through a unit area during a unit time interval. $D$ is the diffusion coefficient or diffusivity. $\varphi$ (for ideal mixtures) is the concentration. $x$ is position, the dimension of which is length.

In one-dimension case, it can be written as:

$$J_i = -(Dc/RT)(\partial \mu/\partial x) \quad \text{(E-5)}$$

where, the index $i$ denotes the $i$th species, $c$ is the concentration (mol/m$^3$), $R$ is the universal gas constant [J/(K.mol)], $T$ is the absolute temperature (K), and $\mu$ is the chemical potential (J/mol).
Flory-Huggins’ polymer solution thermodynamics theory: the thermodynamic equation for the Gibbs energy change accompanying mixing at constant temperature and (external) pressure [279, 280]:

\[
\Delta G_m = \Delta H_m - T \Delta S_m
\]

(E-6)

where \(\Delta G_m\) is the free energy of mixing, \(\Delta H_m\) is the enthalpy of mixing (heat of mixing) and \(\Delta S_m\) is the entropy of mixing. For miscibility to occur, \(\Delta G_m < 0\).

Flory and Huggins derived the Gibbs free energy for polymer solution:

\[
\Delta G_m = RT \left[ n_1 \ln \phi_1 + n_2 \ln \phi_2 + n_1 \phi_2 \chi_{12} \right]
\]

(E-7)

Where the free energy of mixing is a function of the number of moles \(n\) and volume fraction \(\phi\) of solvent (component 1), the number of moles \(n_2\) and volume fraction \(\phi_2\) of polymer (component 2), with the introduction of a parameter \(\chi_{12}\) to take account of the energy of interdispersing polymer and solvent molecules. \(R\) is the gas constant and \(T\) is the absolute temperature.

The temperature dependence of \(\chi_{12}\) has often been expressed by \(\chi_{12} = a + (b/T)\). \(\chi_{12}=1/2\) represents the critical point, \(\Theta\)- point for phase separation. For \(\chi_{12}>1/2\) the solution has a poor solvent, and for \(\chi_{12}<1/2\) a good solvent. For miscibility to occur, \(\Delta G_m < 0\), otherwise phase separation occurs.

B. 3D-TIPS process at different temperatures:

A complete mechanical description of 3D-TIPS process is not straightforward due to complex multi-dynamic events involved at the same time, including solvent diffusion, polymer solution phase separation, polymer phase separation and PVA preform dissolution at complex interface and geometry of the scaffolds. Nevertheless, the thermodynamic theories above provide guidance to design the process conditions and analyse the structure and properties of the scaffold in correlation to the processing conditions.

When PUU-POSS filled PVA preform was immerged into water, DMAC and water started diffusing toward each other across the walls of PVA preform, and the mixture of solvents becomes a poor solvent for PUU-POSS (\(\chi_{12}\) increased in Equation E-7) as it does not dissolve in water. As the result, PUU-POSS gradually precipitated within the confined printed channels.

During 3D-TIPS process, the main competition needs to be controlled and balanced is between the polymer solution phase separation and dissolution of PVA in order to produce scaffolds with desired quality and properties. The principle of 3D-TIPS at cryo-coagulation of the frozen PUU-POSS solution in ice water, 0°C (CC group) is to reduce the diffusion of two solvents and dissolution of the PVA. The low temperature led to decrease miscibility as the \(T \Delta S_m\) term decreased, thus driving \(\Delta G_m\) to more positive (Equation E-5 and E-6). In the meantime, the low diffusion rate of the solvents resulted in a small concentration gradient (\(d\phi/dx\), in Equations E-3 and E-5) and therefore the slowdown of the phase separation induced more uniform porous structure of scaffold. In addition, the slow dissolution of PVA
preform at low temperature minimize the swell of the scaffold and improved the dimension accuracy of the scaffolds.

Increasing coagulation temperature to room temperature 25°C (RTC+H group) led to increasing miscibility as the $T\Delta S_m$ term increases, thus driving $\Delta G_m$ to more negative values (Equation E-6 and E-7). On the other hand, the increase of diffusion of two solvents (Equations E-4 and E-5) and dissolution of PVA at RT facilitated the coagulation of the PUU-POSS at the interface. The fast precipitation of PUU-POSS and the large concentration gradient ($d\phi/dx$) at the interface contributed to the formation of dense skin of the scaffold and non-uniform porous structure. As the result, the swelling ratio of RTC+H group scaffolds is also high due to the fast dissolution of PVA preform.
3D Printing Defined Thermally Induced Phase Separation Compared with Casting of Elastomer Nanohybrid Scaffolds

1. Introduction

Polyurethanes (PUs) comprise a diverse family of high-performance elastomers ranging from flexible and rigid thermosets and thermoplastics to solutions. They combine the durability and toughness of metals with the elasticity of rubbers, making them suitable in several engineered products. [1] Indeed, PUs are among the most common synthetic polymers employed in medical devices and implants,[2] and have been widely tested for various medical applications due to their superior elasticity and appropriate cell-material interactions.[3] For instance, many stimuli responsive biodegradable PUs have been developed as potential drug controlled release carriers based on temperature [4], pH [5] or pressure changes[6]. Non-degradable PUs are another popular choice to provide long lasting support in dynamic environments for long-term implantation [7,8]. Indeed, they have been a lead candidate in the manufacture of commercial cardiovascular devices such as catheters, pacemakers, vascular prosthesis or heart valves [9] owning to their blood compatibility and excellent durability and versatility. PUs have also been often used as wound dressings due to their good barrier properties and oxygen permeability.[9]

PUs are structurally formed by the chemical reaction of polyols and diisocyanate, resulting in a block co-polymer comprising both soft and hard segments.[10] The hard segment within PU chains acting as reinforcement or confinement are responsible for the permanent shape, has a high glass transition temperature and is semicrystalline or highly ordered, in contrast to the soft segment.[11] The hydrophobic soft and hydrophilic hard segments are thermodynamically incompatible and tend to phase-separate into their own micro-domains, where the soft segment gives the material its elastic properties and the hard segment provides physical integrity. [12] The microphase separation of the hard and soft segments provides their prominent elastic and mechanical properties, and absorbance of physical stress. The ratio of the hard to soft segments and the extent of the microphase separation have also influence over the degree of stiffness softening [13]. However, the magnitude to which the co-polymer phase separates, and thus their properties, depend on the molecular composition and chain extenders, the extent of the hydrogen bonding and the soft/hard segment composition.

Innovations in non-degradable PUs have been made for long-term implantations, such as poly (urea-urethanes) (PUUs) are composed of linkages made out of polyurethane and polyurea compounds, when diamines are used as the chain extender rather than diols.[14] The introduction of highly polar urea linkages into the system enhances hydrogen bonding in the hard segments, resulting in a higher degree of microphase separation and the improvement of the mechanical properties compared to common PUs [15,16]. PUUs have demonstrated controllable self-assembly through phase separation between its alternating soft and hard segment chains. PUUs offer excellent hyperelasticity, are relatively
biocompatible and nontoxic, and elicit manageable low inflammatory responses *in vivo* [17]. All these properties make them suitable to be used as soft implants and scaffolds [18].

Polyhedral oligomeric silsesquioxane (POSS), with a size of 1-3 nm in diameter, are cage-like structures composed of an inorganic framework of silicon and oxygen, which is externally covered by organic substituents.[19] While these surface moieties are usually hydrocarbons, they can embody a range of polar structures and functional groups with improvements over the thermal [20], biological [21] and mechanical stability [22] of the reinforced material. PUUs terminated by POSS nanocages (PUU-POSS) have been widely investigated by several groups as coatings [23] and scaffolds for soft tissue engineering, spanning a wide range of tissue applications from bone, cartilage, heart, nerve, bypass grafts to lacrimal ducts. [24]

The fabrication of multifunctional scaffolds is vital to direct 3D tissue ingrowth for the repair of complex multi-tissue defects, where specific surface topography, microarchitectures, and extensive vascular networks are required to influence cellular processes such as adhesion, proliferation or differentiation. Conventional techniques of scaffold fabrication [25] include freeze-drying, solvent casting and practical leaching, gas foaming or electrospinning. Limitations vary from high energy consumption to time consuming, long-term time scaling, use of toxic solvents, low reproducibility or the difficulty to obtain complex structures with tunable nano/micro- and macroscales [26,27]. Improved control over the porosity (i.e. morphology, size, distribution and interconnectivity), and surface topography, can be obtained by tightly controlling the processing conditions and exchanged temperature by means of thermally-induced phase separation (TIPS) [28–30]. Several porous scaffolds for soft tissue engineering applications fabricated by TIPS have been investigated over the years [31,32] from a wide range of polymers. However, their translation into 3D structures remains an issue. The advent of new technologies, such as rapid prototyping, has made it feasible to fabricate scaffolds with tuned internal geometries and controlled porosities as a way to build better artificial constructs [33,34]. They offer precise spatial control with high resolution, uniformity in the interconnectivity of the pores, higher accuracy and greater shape complexity, along with the high speed of printing. Among the various prototyping techniques, micro-extrusion of polymers and hydrogels [35] permits the fabrication of 3D structures in a layer-by-layer fashion with high reproducibility and ease. The scaffold porosity can be easily tailored by design, and gradients of various porosity can be introduced to promote specific cell migration into the core.[36] However, extrusion-based 3D printing does not provide high dimensional precision, and the printing resolution, pre-designed porosity and architectural control are highly dependent on the selected material and on the nozzle diameter.[37] In particular, the use of biocompatible and biodegradable materials is difficult since they are limited to those whose viscosity is sufficiently low that they can be extruded, but high enough so that their shape and architecture are maintained.[38]

PUU-POSS is difficult to print due to its solution condition as synthesized in dimethylacetamide (DMAC) solvent with high boiling temperature, and the slow coagulation process in water. Its high molecular weight also constrains its flow ability in bulk, making it impossible to extrude into filament for extrusion printing. One way around this is by reverse rapid prototyping, in which a negative mold is printed from
a sacrificial material, and the desired polymer is casted out of the mold via a drying process.[37,39] For instance, Hernandez-Cordova et al. had previously casted a PU solution within a poly (vinyl alcohol) (PVA) mold in a vacuum oven to prepare scaffolds for cardiac tissue engineering. [40] However, conventional casting of polymer solutions is limited to developing thin layers of sheets and it is difficult to manufacture large thick constructs. Since the polymer is condensed by bulk solvent evaporation, there is no formation of sub-micron to nano- pores and less control over the topographic features. Improved control over the hierarchical porosity of the scaffold can be obtained if the indirect additive manufacturing technique is combined with a conventional fabrication method. [41–44]

The combination of the widely used, low-cost fused depositing modelling (FDM) technique with conventional TIPS is a good alternative. [45–47] Indeed, the TIPS process has been recently upscaled into 3D-TIPS in combination with 3D printing to overcome the limitations of manufacturing constructs with thick walls and complex geometries, providing a wider hierarchy of uniform interconnected pore structures and controlled topography to the object [18].

In this study, non-degradable PUU-POSS scaffolds are manufactured by means of 3D printed preform confined thermally-induced phase separation (3D-TIPS) or by conventional casting (3D-iCasting) within 3D printed water soluble PVA preform shells used as negative sacrificial molds. The generated scaffolds were characterized for their physico-chemical and mechanical properties. Following this, an assessment of their biological performance using human dermal fibroblasts was carried out.

2. Materials and Methods

2.1 Synthesis of PUU-POSS

Poly (urea-urethane) terminated by polyhedral oligomeric silsesquioxane (PUU-POSS) (as shown in Chapter 1, Figure 1.2) polymer solution in dimethylacetamide (DMAC) was synthesized as needed, using a previously described protocol[48].

2.2. Scaffold manufacturing

2.2.1 Design and fabrication of a PVA preform with a digitally controlled macropore structure

General descriptions were previously referred in Section 2.2.2 Chapter 2

2.2.2 3D printing preform confined thermally-induced phase separation (3D-TIPS) and conventional casting (3D-iCasting)

The PUU-POSS solution was injected into the PVA preform shells through a surface puncture hole and left to sit in a fume hood for 5 min allowing for any air bubbles to rise to the surface. The polymer filled preforms were then subjected to two different temperature exchange processes: confined thermally-induced phase separation (i.e. 3D-TIPS), or confined investment casting (i.e. 3D-iCasting). For 3D-TIPS scaffolds, general descriptions were previously referred in Section 2.2.3 Chapter 2. For 3D-iCasting scaffolds, PUU-POSS was solidified in the PVA preform shells by evaporating DMAC solvent at 70°C in a vacuum oven for up to 7 days.

2.3 Physico-mechanical characterization of the scaffolds
2.3.1 Morphology, structure and surface topography

The scaffold internal architecture, morphology and structure were examined by a field emission scanning electron microscope (Zeiss Supra 35VP FE-SEM). Samples were mounted on aluminum stubs and gold coated (SC500 EMScope) prior to visualization at 5kV.

Changes in the surface topography were analyzed on a Bruker Multimode 8 microscope with a Nanoscope V controller (Bruker Ltd.; Santa Barbara, USA) operating in peak force tapping mode to measure roughness and DMT modulus at the interface. Samples (n=3) were mounted onto metallic AFM substrate discs and topographic micrographs were scanned at 5 µm × 5 µm, 2 µm × 2 µm and 500 nm × 500 nm surface areas. Analysis of the surface roughness and surface modulus was carried out using Nanoscope analysis software (Bruker Version 1.7; Santa Barbara, USA).

The phase transition of the scaffolds was evaluated using a differential scanning calorimeter (DSC Q1000, TA Instrument). Scans were performed between −70°C to 230°C at a scanning rate of 10°C.min⁻¹. The phase composition was examined by X-ray diffraction (XRD Bruker D8 Advance, Germany), with the data collected in the range of 2θ = 5–70° with a step size of 0.03°.

A mercury intrusion porosimeter (Quantachrome Poremaster 60GT, UK) was used to characterize the distribution and structure of the pores of scaffolds (n=6 replicates, freeze-dried in liquid nitrogen).

2.3.2 Apparent volume

Dimensions of the scaffolds (n = 6) as produced were measured manually using a Vernier caliper, and the shrinkage in volume was calculated with respect to the original 3D printed PVA preform.

\[
\text{Shrinkage} \% = \frac{V_{\text{preform}} - V_{\text{scaffold}}}{V_{\text{preform}}} \times 100
\]

2.3.3 Mechanical characterization

General descriptions were previously referred in Section 2.3.1.1 Chapter 2

2.3.4 Surface wettability and protein adsorption

General descriptions were previously referred in Section 2.3.4.4 and 2.3.4.5 Chapter 2

2.4 Biological characterization of the scaffolds

Scaffolds (n=4 replicates; 11 mm diameter and 1.5 mm thickness) for in vitro cell culture studies were sterilized in 70% v/v ethanol for 30 min, air-dried in a sterile cell culture hood, washed in sterile PBS, placed into 48-well plates and pre-incubated for 24 h overnight in 500 µL of Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic (50 µg/mL streptomycin, 50 µg/mL penicillin).

Primary human dermal fibroblast (HDF) cells derived from the dermis of normal human neonatal foreskin or adult skin (HDF 106-05a; Culture Collections, UK) cultured in supplemented DMEM were used throughout the cell culture studies.

2.4.1 Cellular proliferation and viability
Scaffolds were seeded with HDFs in seventh-passage (P7) at a density of $9 \times 10^4 \text{ cells.cm}^{-3}$ in 500 µL of cell culture medium. Media was replaced every three days, and the metabolic activity ($n=4$) was monitored on days 1, 3, 7, and 10 through alamarBlue® (Serotec Ltd; Oxford, UK) testing according to the manufacturer's instructions. At each time point, total DNA was also quantified ($n=4$) using a fluorescent Hoechst 33258 stain (Sigma-Aldrich, UK). The migration speed rate was expressed as the percentage of area covered per time.

2.4.2 Assessment of sulfated glycosaminoglycan (sGAG) synthesis

General descriptions were previously referred in Section 2.4.6 Chapter 2

2.4.3 Extracellular collagen production

General descriptions were previously referred in Section 2.4.5 Chapter 2

2.4.4 Histological evaluation

For histological evaluation, cell-laden scaffolds were fixed in 4% paraformaldehyde (PFA) in PBS, embedded in paraffin and sectioned with a Leica RM2235 rotary microtome. Gross cell morphology was studied with hematoxylin and eosin (H&E) staining. Differential staining of collagen and connective tissue production was evaluated with Van Gieson's stain (HVG), a mixture of picric acid and acid fuchsin.

2.4.5 Fluorescence staining

General descriptions were previously referred in Section 2.5.2 Chapter 2

2.5 Statistical analysis

All results were presented as standard deviation (SD, error bars) of the mean values, and performed at least in triplicate. Statistical analysis of the results was carried out using GraphPad Prism 6 (San Diego, USA) by two-way ANOVA (two independent variables) with Tukey's post hoc test for multiple comparisons. Two-tailed unpaired Student's t test was used when involving one independent variable and only two groups. A value of $p<0.05$ was considered statistically significant.

3. Results and Discussion

Two sets of reversely 3D printed scaffolds were manufactured out of a PUU-POSS solution (Figure 1) based on cryo-coagulation (3D-TIPS) or bulk solvent evaporation (3D-iCasting) using water soluble PVA preforms.

3.1 Effect of the processing technique on the physico-mechanical properties of the scaffolds

Firstly, the effect of the phase behavior under DSC was assessed (Figure 1A). Scaffolds fabricated by 3D-iCasting exhibited a glass transition temperature (Tg) at $-33.6^\circ$C, with no visible endothermic peaks, which might be indicative of a mostly amorphous starting polymeric structure. On the other hand, 3D-TIPS scaffolds exhibited a Tg at $-31.9^\circ$C, and showed a sharp melting peak at 42.2°C, which can be explained by crystal formation within the soft segment chains of PUU, as well as a smaller broad peak at 179°C due to the melting point of partially ordered hard segment chains [18].

The crystalline structure of the scaffolds was further explored by wide-angle X-Ray diffraction (Figure
1B). XRD analysis for 3D-TIPS revealed two sharp Bragg diffraction peaks at 2θ=19.6° and 22.6° with the inter-planar spacing (d-spacing) of 0.44 nm and 0.39 nm respectively, suggesting lateral distances in the interfaces of crystallized soft segments and/or hard segments [18]. For the 3D-iCasting group, the ordered crystal lattice structure previously exhibited was not present, suggesting an amorphous random nanophase structure comprising three broad amorphous halo peaks: an even broadened halo peak at 2θ=19.6° and two shoulders apparent at approximately 2θ=29.8° and 2θ=61.9°. As demonstrated by DSC and XRD analyses (Figure 1A-B), the only difference lies in the polyurethane soft segment chains being crystallized in 3D-TIPS and packed together into a 3D ordered structure.

Scaffolds fabricated by 3D-TIPS shrunk in volume up to a 0.9% compared to the directly printed PVA preform, while those fabricated by 3D-iCasting exhibited a 38% volume shrinkage. These results indicate that the conventional casting technique is only suitable to develop thin layer-like sheets due to the large shrinkage resulting from bulk evaporation and solidification of the liquid phase of the polymeric solution. The 3D-TIPS technique, on the other hand, allows for easy manufacture of large thick constructs with a more faithful representation of the original dimensions of the PVA preform. To tackle the effect of the large shrinkage observed in as-produced 3D-iCasting scaffolds, slightly larger PVA preform shells were printed (i.e. based on the abovementioned shrinkage) for such condition compared to those for 3D-TIPS, so that the final dimensions of the as-produced scaffolds of each condition would be similar for comparison purposes.

The average pore diameter and pore size distribution of the scaffolds was also evaluated (Figure 1C). Analysis with the mercury porosimetry confirmed full interconnectivity for both scaffold groups. A trimodal distribution of the pores was estimated for 3D-TIPS, with distinct three predominant broad peaks [18]: a complex of macropores ranging between 400 to 1 μm (with a relative pore volume of 58.5% and contributing to 1.5 m²/g of relative pore surface area), constrained to the 3D printer nozzle diameter and CAD design; a matrix containing micropores ranging between 1000 to 100 nm (22.1% of relative pore volume and 48.7 m²/g of relative pore surface area); and a further internal hierarchical structure at the nanoscale with smaller pore sizes ranging between 100 to 3 nm (19.4% of relative pore volume and 8.3 m²/g of relative pore surface area). Indeed, the long tail distribution attributed to the micro/nano-porous structure of the 3D-TIPS contributed to its high pore surface area (overall of 58.5 m²/g). In contrast, the pore size distribution of the 3D-iCasting group was mainly monomodal with a predominant broad peak in the distribution function relating to the macropore network structure ranging from 400 to 1 μm (100% of relative pore volume and 1.3 m²/g of relative pore surface area). This is due to the polymer being condensed by confined solvent evaporation with no formation of sub-micron to nano-pores, resulting in its much lower pore surface area instead.

High resolution SEM was used to visualize the macro- and micro-pore structure of the as-produced scaffolds (Figure 1D-I). While a smoother surface was achieved with 3D-iCasting (Figure 1G-I) as seen under SEM, 3D-TIPS exhibited a relatively uniform porous bead-like topography with greater surface area to volume ratio (Figure 1D-F). To quantify the surface modulus and surface roughness of the scaffolds, which are known to affect cell–material interactions [49,50], their surface topological profiles were evaluated by AFM (Figure 2A-F). The surface profiles indicated that 3D-iCasting scaffolds
exhibited relatively smooth surfaces with significantly lower surface moduli (p<0.001) compared to those shown by 3D-TIPS (Figure 2G-H), confirming the observations under SEM. These results corroborate that the roughness of the scaffolds increased with phase separation and crystallization of the polymer solution during confined cryo-coagulation by 3D-TIPS.

Figure 1 Crystalline structure, pore size distribution, morphology and nanophase structure of 3D-TIPS vs 3D-iCasting PUU-POSS scaffolds. (A) Differential scanning calorimetry (DSC). (B) X-ray diffraction (XRD) analysis. (C) Pore size and size distribution. (D-I) Representative SEM images demonstrating variations in the scaffold structure for (D-F) 3D-TIPS and (G-I) 3D-iCasting samples at low and high magnification.
Figure 2 Representative AFM topological profiles of (A-D) 3D-TIPS and (D-F) 3D-iCasting scaffolds at various scanned areas. (G-H) Average surface roughness and surface modulus of the scaffolds (n=3) over different scanned areas. The differences between the experimental groups were analyzed by two-tailed unpaired Student's t test. ***p<0.001; ****p<0.0001.

In this sense, control at the macroscale was achieved by an indirect method of fused deposition modelling that may allow to create complex interconnected structures [38,51], while the micro and nanoscale control was obtained by confined cryo-coagulation (3D-TIPS) of the polymeric
nanocomposite solution. Several authors have indicated that besides porosity, interconnectivity and surface area to volume ratio are important parameters to determine the biological outcome of a scaffold [52,53]. In this study, we minimize the effect of the scaffold macro- pores by designing them to be equivalent in shape and area and focus only on the impact of the processing technique (3D-TIPS vs 3D-iCasting).

Tensile and compressive mechanical tests were performed (Figure 3). Despite the hierarchical porous structure of the cryo-coagulated 3D-TIPS scaffolds (Figure 1C), they exhibited outstanding hyperelastic mechanical behavior with tensile modulus (0.98 MPa), strength (1.33 MPa), ultimate strain (179%) and toughness (137 J. m$^{-3}$x10$^4$). Bulk PUU-POSS polymer exhibited hyperelasticity (i.e. entropic elasticity) with a larger tensile strain at breaking point (332%), tensile modulus (2.26 MPa), strength (2.01 MPa) and toughness (289 J. m$^{-3}$x10$^4$). As expected, the tensile mechanical properties (Figure 3A-D) obtained by 3D-TIPS were significantly (p<0.01) lower compared to those achieved by 3D-iCasting scaffolds. These results indicate that the higher micro/nano- porous structure achieved by phase separation of the polymer via 3D-TIPS confers lower tensile mechanical properties, which is due to the increased content of porosity within the structure [53]. On the contrary, significantly greater (p<0.01) compression modulus and compression strength were obtained for 3D-TIPS scaffolds (0.48 MPa and 0.33 MPa respectively) compared to 3D-iCasting (0.30 MPa and 0.28 MPa respectively) (Figure 3E-F), suggesting that the presence of a hierarchical porous structure is directly correlated with an increase in the compressive mechanical properties [54,55].
Figure 3 Mechanical characterization of PUU-POSS scaffolds fabricated by 3D-TIPS and 3D-iCasting. Comparison of the tensile mechanical properties (n=6): (A) tensile modulus (at 50% strain), (B) ultimate tensile strength, (C) strain at break, and (D) toughness. Comparison of the compression mechanical properties (n=6) (at compression strain 25%): (E) compression modulus, and (F) compression strength. **p<0.01. The differences between the experimental groups were analyzed by two-tailed unpaired Student's t test. **p<0.01.

3.2 Effect of the processing technique on the biological properties of the scaffolds

HDF cells were seeded on 3D-TIPS and 3D-iCasting scaffolds to evaluate their ability to support cell adhesion, cell migration and cellular proliferation over a 10-day period (Figure 4). In this regard, the contact angle of the surface and the ability of the substrate to adsorb protein were evaluated (Figure 4A-B). Indeed, wettability of the substrate and protein adsorption are important parameters for cell attachment and growth [56].

Analysis of the contact angle measurements confirmed that wettability of 3D-TIPS scaffolds exhibited a 2-fold decrease following the highly micro/nano-porous structure acquired via 3D-TIPS compared to 3D-iCasting scaffolds. The highly uniform hierarchical micro/nano-porous structure obtained during the
cryo-coagulation process of 3D-TIPS has been reported to act as a capillary absorbing water, reducing the contact angle [18] of a hydrophobic polymer such as PUU-POSS. In correlation with the contact angle, protein adsorption on 3D-TIPS was significantly (p<0.001) higher compared to 3D-iCasting. Indeed it has been reported that the amount of protein adsorbed increases at relatively higher surface roughness in PU films [57]. A similar trend was observed in terms of cellular behavior, with 3D-TIPS scaffolds exhibiting significantly (p<0.01) higher proliferation (Figure 4C) and metabolic activity (Figure 4D) of HDFs at all day points, as well as promoting greater (p<0.01) cellular proliferation speed rates (Figure 4E). This correlates with the surface roughness and crystalline structures produced through temperature controlled phase separation during 3D-TIPS, and supports the fact that cellular proliferation is maximized on substrates with densely packed and sharp peaks, which could be in part to greater cellular adhesion on substrates of moderate roughness [58]. In combination with the higher micro/nano-porous structure exhibited by 3D-TIPS, greater amount of protein was adsorbed on the substrate at the expense of minor imparted tensile properties, but still comparable to PUUs [3] and to what has been reported by other groups using similar elastomer nanocomposites [59].

Figure 4 Biological activity of PUU-POSS scaffolds fabricated by 3D-TIPS and 3D-iCasting: (A) surface wettability (n=4), (B) protein adsorption (n=4), (C) cellular proliferation metabolic activity (n=4), (D) total DNA analysis (n=4), (E) cellular proliferation speed rate (n=4), (F) GAG synthesis (n=4), and (G)
extracellular collagen (types I-V) (n=4). (H-I) Cellular morphology at day 3 as observed by SEM. (J-K) Histological images of the cross-section of the scaffolds at day 3 stained with Hematoxylin and Eosin (H&E) and Van Gieson (HVG). The differences between experimental groups were analyzed by two-way ANOVA or two-tailed unpaired Student's t test. **p<0.01; ***p<0.001.

The content per cell of produced extracellular collagen and sulfated glycosaminoglycans were next examined over a 10-day period (Figure 4F-G). The ECM is an active and complex component in tissue activity capable of influencing cell survival, proliferation and function [60,61], and any of its components are attractive targets in tissue engineering applications. ECM production is indeed an important function of anchorage dependent cells [62,63], and dermal fibroblasts are intimately linked to the ECM, capable of influencing their migration, gene expression and differentiation capability to other cell types [64]. In particular, collagen and glycosaminoglycans are two ECM components predominantly produced by growing HDF cells [60]. In this regard, similarly to the metabolic and proliferation results, the extracellular collagen and sGAGs content per cell remained significantly (p<0.01) higher on 3D-TIPS scaffolds at all day points compared to cells grown on 3D-iCasting scaffolds.

Cellular morphology of human fibroblasts was observed at the early stages of the cell culture under SEM (Figure 4H-I), exhibiting flat cell bodies and early formation of spindle-like shapes typical of fibroblasts. Histological cross-sections at the early stage of cell culture confirmed cellular infiltration within the two scaffold groups (Figure 4J-K). However, it is of note that the lack of a hierarchical porous structure at the multiscale in 3D-iCasting samples (Figure 1C) makes it difficult to allow for proper cell migration within the polymer core, as shown by reduced staining (Figure 4K) compared to 3D-TIPS samples (Figure 4J). Due to the low porosity on the dense skin-like surface of 3D-iCasting scaffolds, cells are more likely to be prone to delamination [65]. H&S and HVG confirmed greater differential staining of collagen and connective tissue formation in 3D-TIPS scaffolds, in agreement with the sGAG and extracellular collagen quantification assays (Figure 4F-G).

Cellular organization within the scaffolds was qualitatively investigated using immunohistochemistry and confocal microscopy methods (Figure 5). Cells seeded on the scaffolds spread and proliferated until a confluent layer covered the entire surface of the material and confirmed that an open architecture with digitally defined macro-pores facilitates cell infiltration. Confocal images showed a constant increase in cell density from days 3, 7 to 10, but the micro/nano- porous structure and surface topology achieved via 3D-TIPS resulted in higher fluorescent intensity compared to 3D-iCasting as shown by 3D reconstructions of fluorescent intensity. Indeed, by further controlling at different temperatures the phase transition, phase separation, micro-phase separation and reverse self-assembly of the nanocomposite solution in 3D-TIPS scaffolds, different topography levels and micron/nano- hierarchic structures can be induced, likely to impart various degrees of biological activity [18,46]. More to this point, dynamic thermoresponsive stiffness softening has been recently reported on these cryo-coagulated 3D-TIPS scaffolds when subjected at body temperature, driven by two stages of thermodynamic phase transition and local self-assembly close to the melting point of the crystalline domains of the soft segments of PUU. Several in vitro and in vivo studies using 3D-TIPS scaffolds have proven the synergic role of stiffness softening and the hierarchical micro/nano- porous structure in
modulating cell proliferation and differentiation of several cell lines [17,18,65,66]. 3D-iCasting scaffolds lack such thermoresponsive behavior since there is no chain relaxation process influenced by temperature changing conditions during the manufacturing process.

**Figure 5** Confocal microscopy showing HDF proliferation over a 10-day period on PUU-POSS scaffolds fabricated by (A-I) 3D-TIPS or (J-R) 3D-iCasting, with 3D reconstructions of fluorescent light intensity
(G-I, P-R). Actin fibers were stained in green with the nuclei counterstained in blue. Scale bar 200 μm (∗10 magnification) and 100 μm (∗20 magnification).

4. Conclusions

A hybrid elastomeric nanocomposite solution has been reversely 3D printed into scaffolds with control geometry, topography and complex hierarchy based on the processing condition and exchanged temperature. The findings presented here demonstrate that controlled crystallization and phase separation of the polymer results in a complex hierarchical structure at the macro-, micro- and nano-levels as shown by SEM and porosimeter studies of cryo-coagulated 3D-TIPS scaffolds. These were compared against similarly produced 3D-iCasting scaffolds obtained by bulk evaporation, showing that crystalline structures produced through temperature-controlled phase separation of the nanocomposite during cryo-coagulation led to a more textured surface topology and significantly (p<0.01) higher compressive properties. Higher hydrophilicity and greater amount of protein adsorbed were also observed on 3D-TIPS scaffolds compared to 3D-iCasting. These correlate with significantly (p<0.01) greater biological response of 3D-TIPS in terms of metabolic activity and proliferation rates of seeded HDF cells, as well as collagen and GAG deposition content per cell compared to the biological activity exhibited by 3D-iCasting scaffolds.

In contrast to 3D-iCasting for which only thin layer sheets can be produced with a dense skin-like morphology, 3D-TIPS scaffolds with controlled topography and hierarchical porous structure have important implications in controlling and tuning cell-material interactions of grafts and implants for tissue engineering and regenerative medicine applications. The infill density of the structure could be varied to gradually influence the mechanical properties of the construct, as well as controlling the crystallization degree and temperature process at which phase-separation, phase transition and self-assembly of the polymer occurs.

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Reversely 3D printed patterns of a thermoresponsive elastomer guiding cellular behaviour

Curvature driven effects on cellular proliferation and viability

Protein adsorption on the scaffolds was investigated prior to their cell seeding (Figure 1B). In particular, the honeycomb scaffold adsorbed the greatest amount of protein compared with the rest of the scaffold groups (p<0.05), except for the circle pattern for which non-significant differences were found. The amount of protein adsorbed on the honeycomb scaffold was quantified to be nearly a 2-fold increase compared with the triangle pattern, which exhibited the lowest protein adsorption among all samples tested.

HDF cells were then seeded onto the various scaffolds; after a 10-day period of culture, the cellular viability and proliferation were found to be significantly greater for the honeycomb and circle scaffolds with respect to the rest of the groups (p<0.01) (Figure 1 C-D). While the metabolic activity and proliferation of cells seeded on the triangle scaffold increased over time, this scaffold group exhibited reduced cellular activity compared to the rest of the sample types. A similar trend was shown in terms of the extracellular collagen deposition and synthesis of sulfated glycosaminoglycans per cell content (Figure 1 E-F). Indeed, very little extracellular collagen was synthesized by HDF cells on the triangle scaffold, with no major changes among the 10-day period. Cellular proliferation speed rate was also studied over a 7-day period (Figure 1 G). Results confirmed that after 7 days, the proliferation rate was significantly (p<0.01) greater on the honeycomb pattern (2 mm²/h) compared to that in the circle scaffold; followed by the rest of the groups with significantly lower speed rate (p<0.001). In particular, the triangle pattern exhibited reduced proliferation speed rate even after 7 days of culture (0.3 mm²/h).
Biological activity of 3D-TIPS scaffolds (n=4 replicates) with various interconnected macropore geometries: (A) pore size distribution; (B) protein adsorption; (C) alamarBlue fluorescence assay; (D) total DNA analysis; (E) sulfated glycosaminoglycan synthesis per cell content; (F) extracellular collagen deposition per cell content; (G) cellular proliferation speed rate. The differences between the experimental groups were analyzed by one-way or two-way ANOVA. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Curvature driven effects on cellular organization

Cellular organization within the various scaffolds was qualitatively investigated over a 10-day period using immunohistochemistry and confocal microscopy methods (Figure 2–3).
3D reconstructions of fluorescent intensity showed a constant increase in cell density from days 3, 7 to 10 across the thickness of all scaffold groups (Figure 2–3). In particular, the complete thickness of the scaffold was covered in cells at day 10 for the honeycomb pattern, followed by the circle and orthogonal geometries. The triangle pore geometry showed reduced activity as seen from the 3D fluorescent intensity reconstructions.
Figure 2 Representative confocal microscopy images and 3D reconstructions of fluorescent light intensity showing HDF proliferation on days 3 and 7 for different macropore patterns: (A) honeycomb; (B) circle; (C) orthogonal; (D) rectangle and (E) triangle geometries. Scale bar 200 μm (x10 magnification).

Figure 3 Representative confocal microscopy images and 3D reconstructions of fluorescent light intensity showing HDF proliferation on day 10 for the various scaffold patterns. Scale bar 200 μm (x10 magnification) and 100 μm (x20 magnification).
**Appendix. D**

**Thermoresponsive Stiffness Softening of Hierarchically Porous Nanohybrid Membranes Promotes Niches for Mesenchymal Stem Cell Differentiation**

**Fabrication of membranes:** Porous membranes with different stiffness were fabricated following an adapted protocol of TIPS at different thermal conditions (Table 1). Briefly, a POSS-terminated PUU polymer solution was poured onto a square-shaped glass mold (100 mm × 100 mm × 500 μm in terms of width, length and height) and coagulated at different temperatures according to reference to allow for solvent exchange, resulting in three different scaffold groups: CC (cryo-coagulation), CC+H (cryo-coagulation and heating), and RTC+H (room temperature coagulation and heating).

**Table 1** Processing conditions of TIPS

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>PUU-POSS solution poured on glass mold</th>
<th>Coagulation conditions</th>
<th>Thermal treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature coagulation + heating (RTC+H)</td>
<td>N/A</td>
<td>25°C water for 24 h</td>
<td>40°C water for 3 h</td>
</tr>
<tr>
<td>Cyro-coagulation (CC)</td>
<td>-20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
<td>No thermal treatment</td>
</tr>
<tr>
<td>Cyro-coagulation +heating (CC+H)</td>
<td>-20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
<td>40°C water for 3 h</td>
</tr>
</tbody>
</table>

**Results and Discussion**

Elastomer membranes of PUU with chain ends terminated with POSS nanocage were fabricated following a TIPS process on a flat glass mold. Three different thermal processing conditions were developed in parallel to an inversely 3D printed protocol reported recently as comparison, summarized in **Table 1** in Methods, rendering membranes with differential starting stiffness...
and porous structure. Three different scaffold groups were developed: cryo-coagulation (CC), cryo-coagulation and heating (CC+H), and room temperature coagulation and heating (RTC+H).

Tunable stiffness softening with hierarchical porous structures by TIPS

The membranes made at the three phase separation conditions behaved differently under tensile stress (Figure 1). Despite the highest porosity (89%), CC membranes possessed outstanding hyperelastic mechanical behavior with the highest tensile modulus (20.0 MPa), strength (20.7 MPa), ultimate strain (711%) and toughness (767 J. m\(^{-3}\times10^4\)), compared with CC+H and RTC+H (Figure 1A-C). Similar to the membranes made by reverse 3D printing, pronounced stiffness relaxation was also observed in the CC group at body temperature (37°C) (Figure 1D-G). After a 28-day period of isothermal relaxation, a decrease in all mechanical properties (Figure 1A-G) was exhibited, especially within the CC scaffold group, with a significant reduction of the tensile modulus (62%) and strength (82%) respectively (p<0.001); after 35 days incubation, all groups reached similar values (p-value non-significant), reminiscent of their ‘stiffness memory’ effect in 3D-TIPS scaffolds. It is of note that, after stiffness softening, the tensile moduli of all the TIPS membranes reduced to about 2-3 MPa (Figure 1D), which is in the similar level of cartilage, higher than those of 3D-TIPS scaffolds with additional larger macro-pores introduced by 3D printing (0.3 to 1.0 MPa).

The stiffness softening was accelerated at dynamic cyclic tensile loadings (i.e. 200 cycles) with a fixed strain at 25% before and after isothermal relaxation up to 35 days (Figure 1H-J). While it was evident that both the CC and CC+H membranes became softer with increasing reversible compliance, the RTC+H group did not exhibit too much change. The continuous softening and memory of the hyperelastic rubber phase were tracked when subjected to high cyclic numbers up to 2×10^6 times at 37°C (Figure 1K-M). As the number of cycles increased, the pronounced damping and reduction of the load amplitude and hysteresis loop area were evident in all samples of CC membranes, and a small trace of stiffness relaxation in CC+H was also detected, compared to RTC+H. A wider spectrum of relaxation times was associated with the CC group compared to the rest of the sample groups. After 35 days, all membranes relaxed to similar hyperelasticity, showing reversible and linear stress and strain profiles with little hysteresis energy loss measured throughout the prolonged cycles, confirming the ‘stiffness memory’ effect of the membranes (Figure 1N-P).
Figure 1 Stiffness softening produced by TIPS at three processing conditions (CC, CC+H and RTC+H). (A-C) Representative stress-strain curves. (D-G) Tensile mechanical properties before and after incubation >35 days at 37°C for tensile modulus, ultimate tensile strength, toughness and strain at break (n=6). (H-J) Dynamic cyclic tensile loading at 0-200 cycles before
and after 35 days (n=2); (K-M) dynamic tensile loading at increasing cycles at day 0 (n=2); (N-P) dynamic tensile loading at increasing cycles at day 35 (n=2). The differences between the experimental groups were analyzed by two-way ANOVA with Tukey's post hoc test, or two-tailed unpaired Student's t test. ****p<0.0001; *p<0.05; n.s = non-significance.

All scaffold groups exhibited a hierarchical porous structure spanning a wide range of scales from macro-, micro- to nanometers (300 μm to 0.1 nm), but with different size distributions. The average pore diameter and pore size distribution of the three different scaffold groups were compared by mercury intrusion porosimetry (Figure 2A-C) and electron microscopy (SEM) (Figure 2D-I). The CC scaffold exhibited the widest hierarchy of pore size distribution but with predominant micro- to nano- pores (84% of pore size <10 μm), hence the overall highest porosity (89%) and surface area (160.86 m².g⁻¹) (Figure 2A) as a result of a slow coagulation at the liquid-solid interface between water and the frozen polymer solution. There was a slight shrinkage (82% porosity and 155.78 m².g⁻¹ surface area) after incubation for 28 days at 37°C. This is further supported by the relatively uniform porous bead-like morphology from the top surface throughout the whole cross-section in CC membranes due to the cryo-process, as seen under SEM at different magnifications (Figure 2D, G). The CC+H scaffold presented a slightly smaller porosity (80%) to that of CC with some decrease of the pores at the micro- and nano-scales (80% pores <10 μm), and thus surface area (128.17 m².g⁻¹) (Figure 2B) due to shrinkage resulting from the post-thermal treatment. Those beads appeared to be fused with less nano-pores due to the shrinkage (Figure 2E, H). The RTC+H group exhibited the lowest porosity (71%) with a significant reduction of pores at micro/nano- meters (only 49% pores <10 μm), thus the lowest pore surface area (49.92 m².g⁻¹) (Figure 2C). A dense skin-like surface of the membrane was generated at the liquid-liquid interface between water and the polymer solution and non-uniform macro- pores under skin across the whole thickness of the membrane were produced by a faster coagulation at room temperature (Figure 2F, I).
Figure 2 Hierarchical structure of ‘stiffness memory’ PUU-POSS membranes by TIPS at various phase separation conditions (CC, CC+H and RTC+H), before and after 28 days incubation in vitro at body temperature. (A-C) Pore size and size distribution. (D-I) SEM micrographs demonstrating morphology and porous structure at the (D-F) top-surface and (G-
I) cross-section. (J-O) HRTEM images of the membranes at day 0 (J-L) and after 28 days (M-O) in vitro incubation (insets of electron diffractions). (P) Schematic of phase transition of the nanophase structure before and after stiffness softening of the membranes in vitro.

XRD spectra (Table 4) and high resolution TEM (Figure 2J-O) shed more insight on the stiffness softening mechanism. The phase transition from semicrystalline domain to amorphous rubbery soft domain is the driving force for stiffness softening. HRTEM images (Figure 2J-O) verified the phase transition and evolution of the nanophase structure of these membranes before and after incubation for 28 days. The bright crystalline nano-domain of soft segments organized the dark nano-domains of hard segments into a highly ordered nanophase structure in as-produced CC membranes (Figure 2J), which contributed to the overall high mechanical properties (Figure 1). Such ordered structure gradually disorganized into a random nanophase structure of soft and hard segments, with evidence of a diffusion halo from both electron diffraction (Figure 2M) after incubation for 28 days, resulting in stiffness relaxation observed in Figure 1. Figure 2K showed a mesophase-like stage of melting crystalline nanophase structure of CC+H membranes after 3 h of thermal treatment at 40°C. RTC+H membranes formed a uniform rubber nanophase structure with hard domains as physical crosslinking points randomly distributed into a continuous soft domain, a typical nanophase structure of thermoplastic polyurethanes (Figure 2L), showing characteristics of hyperelasticity of the elastomer. After incubation for 28 days, all the membranes shared a more or less similar random nanophase structure as shown in Figures 2M-O. Besides, there was a subtle change in the rubber nanophase structure over the time of incubation as indicated by WAXD spectra, with emerging three pronounce broad halo peaks with 20 at around 20°, 29° and 41° (Table 4), suggesting the low-dimensional and short distance chain packing of hard and soft chain segments and their interface during the incubation. Therefore, such nanophase structure is not completely random, named quasi-random nanophase. The phase transition and subsequent reverse self-assembling during stiffness softening echoed a wider spectrum of relaxation times associated with the CC group compared to the other two sample groups, which was revealed by the dynamic mechanical test above.

Like other polyurethane elastomers, PUU-POSS is chemically stable and non-degradable. It is clear that the differences in the measured stiffness (Figure 1 D-G and H-P) and corresponding phase structures (Figure 2D-I and J-O) of the membranes at different processing conditions, incubation and cyclic loading over the time at body temperature, are contributed by the polymer
chain organization and interaction at multiscale. This physical evolution of condensed structure of PUU-POSS elastomer involves chain conformation, nano- phase separation, and phase transition between the semicrystalline phase and quasi-random rubber phase, during the crystallization/melting of the soft segments and self-assembly/inverse self-assembly of both soft and hard segments. Besides, the stiffness softening effect (Figure 1P) could be in principle reversible or partially reversible by re-crystallization or densely packing at a suitable temperature; however, it may be kinetically slow in the solid state.

**Effects of porosity and stiffness softening on hBM-MSCs proliferation**

The surface wettability (Figure 3A) and protein adsorption (Figure 3B) of the scaffold groups were characterized. The CC group demonstrated the lowest contact angle and highest protein adsorption compared to CC+H and RTC+H, which is attributed to its unique surface porous structures at the micro- and nano- scales (Figure 2D, G). Despite the hydrophobic nature of PUU-POSS nanohybrid elastomer, the uniform micro- to nano- porous structure formed at the surface of the CC group acted as a capillary, which absorbed water, thus, increasing the wettability of the surface and protein adsorption. Similar capillary effects took place on CC+H to a lesser extent due to the small shrinkage after the post treatment (Figure 2B and Table 3). In contrast, the RTC+H group showed the highest contact angle and lowest protein adsorption contributed by the formation of the dense surface.

Cells exhibited higher metabolic activity and proliferation rates on the initially soft CC+H scaffold at day one post-seeding, but a significant peak (p<0.01) was reached at day 10 on the CC scaffold. Although non-significant differences were found after a 10-day period between the CC and CC+H group (Figure 3C-D), cell proliferation was accelerated on CC scaffolds where stiffness softening was taking place, while remaining significantly higher than the RTC+H group until confluence (p<0.01). This trend of cellular viability was also visualized by fluorescent phallolidin F-actin staining under confocal microscopy (Figure 3E-M). A distinct difference of cell morphology on the three membranes were observed at the early stages of cell culture, with the most number of MSCs and filamentous actin (F-actin, in green) on CC+H samples and the least on the CC ones (Figure 3 E-G), in agreement with the results of metabolic activity and total DNA (Figure 3C-D). This indicates that the soft surface of CC+H and RT+H promoted more expression of filopodium/lamellipodium that enhanced cell adhesion and migration on the membrane.

On the other hand, MSCs appeared to migrate and proliferate slowly on the stiff surface of CC
samples in the early stage of the cell culture (Figure 3E) despite their most hydrophilic surface and highest protein absorption among the three groups (Figure 3A-B). Nevertheless, the profound stiffness relaxation effect exhibited by CC samples (Figure 1D-G) during the first 2 weeks of incubation appeared to trigger more cellular metabolic activity and accelerated proliferation for a relative longer period of time, coupled with a greater hierarchical micro/nano-porous structure (Figure 2D, G). The highest cellular viability and substantial cellular reorganization on the CC membranes over 10 days while stiffness softening was occurring was confirmed by confocal microscopy (Figure 3K).
Figure 3 hBM-MSC proliferation on stiffness softening porous membranes by TIPS at various thermal conditions (CC, CC+H and RTC+H): (A-B) Wettability ($n=3$) and protein adsorption ($n=3$); (C-D) metabolic activity and cellular proliferation ($n=3$); (E-M) Immunofluorescent staining (F-actin in green and nuclei in blue) over 10 days. The differences between the experimental groups were analyzed by two-way ANOVA with Tukey's post hoc test. **p<0.01, *** p<0.001; n.s = non-significance.
Effects of stiffness softening and porosity on in vitro chondrogenesis of hBM-MSCs

Chondrocyte-like MSCs were highly present on the CC+H and CC membranes (Figure 4A-B), highlighted by Collagen II and Aggrecan markers under a fluorescent confocal microscope at day 28 of chondrogenic differentiation, in contrast to RTC+H (Figure 4C). SOX 9, an important regulator of the chondrocyte phenotype, controls gene expression of COL2A1 (Collagen II), COLX (Collagen X) and ACCAN (Aggrecan), all of which encode important cartilage-like extracellular matrix (ECM) proteins. More to this point, those gene expression markers of chondrogenesis were quantified by qPCR during differentiation towards the chondrogenic lineage (Figure 4D-G). Gene expression activity increased with the culture time in all scaffold groups to different extents, compared to tissue culture plate (TCP) control. Among the various scaffold groups, the CC+H scaffold appeared to promote the highest expression of all chondrogenic markers throughout the 28 days of differentiation. The relative gene expression of ACCAN, SOX9, COL2A1 and COLX in the CC+H scaffold was significantly higher (p<0.001) than the spheroid positive control after 4 weeks of culture. The levels of sulfated glycosaminoglycans (sGAG) per DNA content (sGAG/DNA) were also the highest for the CC+H group among the rest of the scaffold groups (p<0.01) (Figure 4H). It is of note that the gene expression values quantified for the stiffer CC group with similar surface and porosity were lower than those for the CC+H group, but still significantly higher than the softer RTC+H group.

An ELISA technique was used to further quantify the production of sGAG, Aggrecan and Collagen II (Figure 4I-K). After chondrogenic differentiation, higher expression of glycosaminoglycans, Aggrecan and Collagen II was detected on both CC+H and CC membranes compared to the rest (p<0.001 to 0.01), in consistence with the results obtained by qPCR. This data further confirmed that both the CC+H and CC scaffold groups promoted more rapid chondrogenesis of hBM-MSCs, as demonstrated by histological sectioning at week 4 (Figure 5). Increased Collagen II and proteoglycan formation associated with chondrogenesis was observed throughout the whole cross-section of the CC (Figure 5A1-A4) and CC+H membranes (Figure 5B1-B4). More intriguingly, a large number of MSC cells showing chondrocyte phenotype migrated into the lacunae within the bead-like porous network within the CC and CC+H samples, opposed to those only on the top dense surface of the RTC+H membrane.

It was expected that a low distribution of calcium and phosphorous during chondrogenesis was detected by EDX mapping (Figure 6A). The tensile mechanical properties of the membranes
after chondrogenesis differentiation were also compared with cell-free constructs after incubation at 37°C for 35 days (Figure 6B-E). Despite the stiffness softening of the CC and CC+H scaffolds themselves, a substantial increase of the resulting stiffness, strength, ultimate strain and toughness respectively was measured, attributed to cell-derived ECM into the TIPS-induced porous membranes where most chondrogenesis occurred. This is a potentially highly desirable smart cartilage implants/hip implant coatings with high stiffness for providing initial mechanical support and stiffness relaxation for aiding biological tissue remodelling following surgical tissue reconstruction.

The CC+H and RTC+H membranes became softer after post-thermal treatment, but remained with a distinctly different surface morphology, which indicates the influential role of the surface morphology and hierarchical porous structure of the membranes on regulating chondrogenesis of hBM-MSCs. On the other hand, CC and CC+H membranes, with similar surface morphology and porosity, but different initial stiffness and stiffness softening degree, shed more insight about the cellular responses to the stiffness softening mechanism highly exhibited by the CC samples.

Figures 4-5 show that the MSC fate towards chondrogenesis was mainly favored in terms of the initial soft stiffness of the CC+H scaffold coupled to its hierarchical porous structure. The initial high stiffness of the CC membranes appeared to slowdown chondrogenic differentiation compared to CC+H (p<0.01) in the beginning. As more MSCs grew on the surface and inside of the porous scaffold (Figure 3), their differentiation potential was improved and regulated by the ECM derived microenvironment generated by earlier differentiated cells on the substrate with on-going stiffness softening, a similar trend to the MSCs on reversely 3D-printed scaffolds made by 3D-TIPS. Therefore, CC membranes remained efficient chondrogenic differentiation during stiffness softening, significantly higher than RTC+H and both the TCP and positive controls. Histological cross-sections of the cell-laden membranes after differentiation (Figure 5) showed that cartilage-like tissue grew and penetrated into the hierarchically micro/nano-porous structures of both CC+H and CC membranes (Figure 2D-F) and compared with non-cell-laden membrane sections used as negative control (Figure 3 A-B). Unsurprisingly, only a thin layer of stained tissue was observed on the surface of the RTC+H scaffold, prone to be delaminated, but very little within the cross-section due to the low porosity on the dense surface skin (Figure 2F). In short, both CC and CC+H membranes stimulated more chondrogenesis, thanks to a combination of a soft matrix or stiffness softening with appropriate hierarchical porosity that allowed cells to attach, migrate and grow, stimulating cartilage-like integrin
mediators and rendering microenvironment niche for cellular proliferation.

Figure 4 Chondrogenesis of hBM-MSCs on stiffness softening porous membranes (CC, CC+H and RTC+H): (A-C) Immunofluorescent analysis of hBM-MSC under chondrogenic differentiation after 28 days showing Collagen II (blue) and Aggrecan (purple), with F-actin (green) counterstaining. (D-G) Gene expression profile by qPCR over 4 weeks (n=6); comparative analysis for (D) SOX9, (E) ACCAN, (F) COL2A1, and (G) COLX. (H) Synthesis of sulfated glycosaminoglycans during a 4-week period (n=6). (I-K) ELISA of glycosaminoglycans, Aggrecan and Collagen II production (n=6). The differences between the experimental groups were analyzed by two-way ANOVA with Tukey's post hoc test, or two-tailed unpaired Student's t test. **p<0.01; ***p<0.001; **** p<0.0001.
Figure 5 Functional evaluation of chondrogenic differentiation on stiffness softening porous membranes (CC, CC+H and RTC+H): histological images of the cross-section (×4 objective lens) and in-plane (×40 objective lens) of the membranes at week 4 stained with Hematoxylin and Eosin, Alcian Blue, SOX9, and Collagen II.
Figure 6 Element detection and tensile mechanical properties of differentiated cell-laden stiffness softening porous membranes. (A, F) Production of calcium and phosphorous after chondrogenesis and osteogenesis \((n=6)\). (B-E) Tensile modulus (at 50% strain), ultimate tensile strength, toughness and strain at break after chondrogenic differentiation over 28-35 days compared to day 0 and day 35 after stiffness relaxation of cell-free membranes \((n=6)\). (G-J) Tensile modulus (at 50% strain), ultimate tensile strength, toughness and strain at break after osteogenic differentiation over 21-28 days compared to day 0 and day 35 after stiffness relaxation.
relaxation of cell-free membranes \( (n=6) \). The differences between the experimental groups were analyzed by two-way ANOVA with Tukey's post hoc test, or two-tailed unpaired Student's \( t \) test. \( *p<0.05; **p<0.01; ****p<0.0001. \)

**Effects of stiffness softening and porosity on in vitro osteogenesis of hBM-MSCs**

The stiffness softening of the membranes also regulated hBM-MSCs towards the osteogenic lineage in the osteogenic differentiation medium. Immunofluorescent images stained by Collagen I and calcium deposition showed that the most osseous tissue formation occurred on the CC membranes after 21 days, opposed to little calcium presence on either the CC+H or RTC+H samples (Figure 7A-C). The quantification of osseous tissue formation in terms of Alizarin Red and alkaline phosphatase activity, as markers of calcium deposition, confirmed with significantly higher production on the CC scaffolds compared to both the CC+H and RTC+H groups (p<0.0001) after 3 weeks (Figure 7D-E).

The gene expression of key regulators of osteogenesis, such as SP7 (Osterix), COL1A1 (Collagen I), SPP1 (Osteopontin), ALP (alkaline phosphatase), BGLAP (Osteocalcin) and RUNX2 (cbfa-1) gradually increased during in vitro differentiation as evaluated by qPCR (Figure 7F-K). Outstanding osteogenic differentiation of hBM-MSCs occurred on the initially rigid CC scaffold within 21 days; with the highest expression of all genes compared to the rest of the membranes and the spheroid positive control (p<0.0001). In addition, the production of Osteocalcin and Collagen I analyzed by ELISA over a 3-week period (Figure 7L-M) was significantly higher from the CC membranes than the rest (p<0.0001), in consistence with the results by qPCR. Compared to membranes with 3D digitally printed macro-pores, such differences are even higher, indicating the stiffness softening as a predominant drive for promoting osteogenesis.

Osteogenesis after 21 days on the CC scaffold was also confirmed by the histological staining of Collagen I and Alizarin Red for calcium (Figure 8). Deposition of bone-like ECM components associated with osteogenesis was observed predominantly throughout the porous network of the CC scaffold (Figure 8A1.1, A3.1). Calcium deposition on the membranes was also directly detected by EDX analysis (Figure 6F), where CC membranes exhibited the highest accumulation. The tensile mechanical properties of the membranes after osteogenesis were also compared with cell-free constructs after in vitro stiffness relaxation > 28 days (Figure 6G-J). Similar to the chondrogenesis example, substantial enhancements of all the tensile mechanical properties of the CC group after stiffness softening are attributed to cell-derived
produced ECM during the pronounced osteogenesis on the CC group.

Different from the chondrogenesis case studied above, the initial high stiffness and subsequent stiffness relaxation appeared to be predominant factors for promotion of osteogenesis of hBM-MSCs with evidence on the porous CC scaffold. Osteogenesis remained constantly active over the 28-day period (Figure 7), regardless the softer substrate after the first two weeks of incubation at body temperature, indicating the cellular ‘mechanical memory’ of the hBM-MSCs on initial stiff substrates. The proliferation and differentiation of MSCs continued increasing significantly during the subsequent profound stiffness softening of the CC membranes, demonstrating resilient cellular ‘mechanical memory’ regardless the softening substrate. In this case, a gradual shift from the original mechanosensing towards de novo cell-derived matrix sensing in a more physiologically microenvironment niche generated by the cells themselves may have occurred. While differences in the associated bone gene expression in the CC+H and RTC+H membranes remained, the effect of the micro/nano-porous structure is again noticeable (p<0.05), but more significant than those in the scaffolds made by 3D-TIPS with digitally printed macro-pores. Therefore, the influence of the initial stiffness and subsequent stiffness softening of the CC scaffold on modulating osteogenesis is overriding its porosity.
Figure 7 Osteogenesis on stiffness softening porous membranes (CC, CC+H and RTC+H). (A-C) Immunofluorescent analysis of hBM-MSC after 21 days, showing F-actin (green), Collagen I (blue) and calcium (red). (D-E) Alizarin Red S ($n=6$) and alkaline phosphatase activity ($n=6$) after over 21 days. (F-K) Gene expression profile by qPCR over 3 weeks ($n=6$); comparative analysis for (F) ALP, (G) SPP1, (H) COL1A1, (I) SP7, (J) BGLAP and (K) RUNX2. (L-M) ELISA of Osteocalcin and Collagen I production ($n=6$). The differences between the experimental groups were analyzed by two-way ANOVA with Tukey's post hoc test, or two-tailed unpaired Student's t test. **p<0.01; ***p<0.001.
Figure 8 Functional evaluation of osteogenic differentiation on stiffness softening porous membranes (CC, CC+H and RTC+H): histological images of the cross-section (×4 objective lens) and in-plane (×40 objective lens) of the membranes stained with Hematoxylin and Eosin, Collagen I and Alizarin Red.

Elastomeric nanohybrid membranes with thermoresponsive stiffness softening and unique porous structure were developed by thermally-induced phase separation (TIPS). The initial stiffness and subsequent stiffness softening coupled with the interconnected micro/nano-porous structure of the membranes promote niches that regulate the differentiation of human bone-marrow derived mesenchymal stem cells towards the osteogenic and chondrogenic lineages, promising smart scaffolds/coatings with matched mechanical properties for tissue reconstruction and regeneration.
### Table 1 Physico-mechanical properties of stiffness softening porous membranes, before and after relaxation at body temperature ($n=6$).

<table>
<thead>
<tr>
<th>3D-TIPS scaffold</th>
<th>Scaffold Density, kg.m$^{-3}$</th>
<th>Total Porosity, 100%</th>
<th>Tensile modulus @ 50% strain, MPa</th>
<th>Tensile modulus @ 100% strain, MPa</th>
<th>Ultimate Strength, MPa</th>
<th>Strain at break, %</th>
<th>Toughness, J. m$^{-3} \times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>D0</td>
<td>41 (±3)</td>
<td>89 (±2)</td>
<td>20.0 (±1.9)</td>
<td>19.8 (±2)</td>
<td>20.7 (±0.4)</td>
<td>711 (±30)</td>
</tr>
<tr>
<td>CC</td>
<td>D28</td>
<td>60 (±3)</td>
<td>83 (±2)</td>
<td>7.7 (±1.7)</td>
<td>7.2 (±1.9)</td>
<td>3.7 (±1.1)</td>
<td>433 (±35)</td>
</tr>
<tr>
<td>CC</td>
<td>D35</td>
<td>63 (±7)</td>
<td>84 (±7)</td>
<td>3 (±1.7)</td>
<td>2.9 (±1.9)</td>
<td>2.2 (±1.2)</td>
<td>318 (±67)</td>
</tr>
<tr>
<td>CC+H</td>
<td>D0</td>
<td>68 (±5)</td>
<td>80 (±2)</td>
<td>7.3 (±0.9)</td>
<td>7.1 (±1)</td>
<td>5.7 (±0.3)</td>
<td>496 (±32)</td>
</tr>
<tr>
<td>CC+H</td>
<td>D28</td>
<td>73 (±8)</td>
<td>79 (±2)</td>
<td>5.8 (±0.4)</td>
<td>4.3 (±0.8)</td>
<td>2.7 (±0.4)</td>
<td>398 (±41)</td>
</tr>
<tr>
<td>CC+H</td>
<td>D35</td>
<td>75 (±9)</td>
<td>78 (±4)</td>
<td>2.6 (±0.5)</td>
<td>2.9 (±0.7)</td>
<td>2.10 (±0.5)</td>
<td>319 (±42)</td>
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<tr>
<td>RTC+H</td>
<td>D0</td>
<td>90 (±12)</td>
<td>71 (±2)</td>
<td>2.1 (±0.2)</td>
<td>2.2 (±0.5)</td>
<td>2.2 (±0.2)</td>
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<tr>
<td>RTC+H</td>
<td>D28</td>
<td>92 (±10)</td>
<td>70 (±1)</td>
<td>2.1 (±0.7)</td>
<td>2.2 (±0.4)</td>
<td>2.1 (±0.6)</td>
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<td>RTC+H</td>
<td>D35</td>
<td>92 (±9)</td>
<td>71 (±3)</td>
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<td>2.1 (±0.4)</td>
<td>1.9 (±0.3)</td>
<td>298 (±32)</td>
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### Table 2 Hysteresis values (i.e. energy lost) of the various membranes before and after thermal relaxation during tensile cyclic loading in the strain domain ($n=2$).

<table>
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<tr>
<th>Type of test</th>
<th>Day</th>
<th>No. of cycles</th>
<th>Hysteresis energy (J.m$^{-3}$)</th>
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<td></td>
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<td>CC</td>
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<tr>
<td>Tensile</td>
<td>D0</td>
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<tr>
<td></td>
<td></td>
<td>10,000-10,200</td>
<td>63 (±8)</td>
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<tr>
<td></td>
<td></td>
<td>100,000-10,200</td>
<td>41 (±10)</td>
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<tr>
<td></td>
<td></td>
<td>200,000-200,200</td>
<td>19 (±4)</td>
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</table>
Table 3 Pore diameter, pore volume and relative pore surface fraction of stiffness softening porous membranes at day 0 and after 28 days at body temperature *in vitro*.

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<thead>
<tr>
<th>TIPS scaffold</th>
<th>Pore Diameter, nm</th>
<th>Pore Volume, cm³.g⁻¹</th>
<th>Relative Pore Volume, %</th>
<th>Surface Area, m².g⁻¹</th>
<th>Relative Surface Area, %</th>
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<td></td>
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<tr>
<td><strong>CC</strong></td>
<td>1000,000 to 10,000</td>
<td>13.4</td>
<td>16</td>
<td>25.7</td>
<td>16</td>
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<tr>
<td></td>
<td>10,000 to 37</td>
<td>32.1</td>
<td>38</td>
<td>101.2</td>
<td>63</td>
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<td></td>
<td>37 to 5</td>
<td>39.6</td>
<td>46</td>
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<tr>
<td></td>
<td><strong>Total</strong></td>
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<td><strong>160.9</strong></td>
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<td>11.8</td>
<td>20</td>
<td>23.7</td>
<td>18</td>
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<td>10,000 to 37</td>
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<td>37 to 5</td>
<td>18.5</td>
<td>49</td>
<td>25.3</td>
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<td></td>
<td><strong>Total</strong></td>
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<td>37 to 5</td>
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<td>48.7</td>
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<td></td>
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<td><strong>30.6</strong></td>
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<td><strong>CC</strong></td>
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<td>12.6</td>
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<td>16</td>
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<td>10,000 to 37</td>
<td>32.1</td>
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<td>Membranes</td>
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<td>41.29</td>
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</table>

**Table 4** Evolution of XRD peaks of the membranes with ‘stiffness memory’ over 28 days *in vitro* incubation. Degree of crystallinity (Dc, %), d-spacing (d, Å)
Appendix. E

Improved tracheobronchial epithelium differentiation on a thermoresponsive 3D porous elastomer

1. Introduction

Respiratory epithelial cells lining the upper respiratory airways are the primary mechanical and immunological protective barrier between the host and the external environment [1–3]. They constitute a pseudostratified layer made up of different epithelial cell types (e.g. basal, ciliated and secretory) whose relative distribution varies along their anatomic location within the airway tract to fulfil a wide range of integrated functions. These comprise barrier integrity, mucus production, antimicrobial capability, and cilia motility [4]. Tracheal defects or tracheobronchial epithelial associated diseases are largely dependent on a dysfunctional tracheobronchial epithelium, with a delayed epithelium regeneration increasing the risk of infection.

A well-established method to investigate the role of airway epithelial cells and understand their differentiation and regenerative mechanisms following airway epithelium damage are 2D co-culture systems at an air-liquid interface (ALI). However, they do not represent the complexity of the native structure and are just an oversimplified design. The development of 3D structures as in vitro models [5–7] in the last few years have paved the way to generating more accurately screening platforms for oral drug discovery or therapeutic testing, and tissue regeneration [8].

An appropriate barrier function is a key parameter for maintaining cell-material interactions at an ALI, for which minimal porosity is ideally best suited. While semi-closed pores within the scaffold help create a more uniform surface and maintain its integrity, it can also limit nutrients and gas exchange delivery [9]. The physical porosity of membranes used to grow epithelial-like cells at an ALI is rarely reported in literature [10,11], but evidence is that growth and differentiation of epithelial cells on porous membranes is far superior compared to that on solid substrates [12]. Porous scaffolds permit a multidirectional exposure to nutrients and oxygen compared to a depletion of them on solid supports, and since cells grown at an ALI can only obtain nutrients from the basolateral medium, the porosity of the scaffold is an important feature to develop a functional tracheobronchial epithelium.

Hierarchical micro/nano- porous elastomer scaffolds exhibiting thermoresponsive dynamic stiffness softening at body temperature were recently fabricated by a customized thermally-induced phase separation (TIPS) process [13], further developed into 3D-TIPS in combination with 3D printing [14], using a solution-based poly(urea-urethane) terminated by polyhedral oligomeric silsesquioxane (PUU-POSS). Systematic studies have shown the roles of the hierarchical porous structure and dynamic stiffness in modulating growth and proliferation of human dermal fibroblasts (HDFs) [14] and differentiation of human bone-marrow derived mesenchymal stem cells (hBM-MSCs) in vitro [13,15], as well as tissue ingrowth, vascularization and macrophage polarization in vivo [16].
Here, the porosity of these soft thermoresponsive elastomeric nanohybrid scaffolds is modified by introducing a porogen-leaching process during TIPS as a way to create a more appropriate scenario for nutrients and gas exchange. Epithelium differentiation is further enhanced by infusing a collagen type I hydrogel, a predominant component of the respiratory tract [17]. This nanoelastomer-collagen hybrid allows the culture of human bronchial epithelial cells (hBEpiCs) on monoculture conditions, and their co-culture with human bronchial fibroblasts (hBFs) or human bone marrow derived mesenchymal stem cells (hBM-MSCs).

2. Materials and Methods

2.1 Fabrication and characterization of thermoresponsive stiffness scaffolds

2.1.1 Scaffold fabrication

3D porous elastomer scaffolds from a poly (urea-urethane) (PUU) solution terminated with polyhedral oligomeric silsesquioxane (POSS) nanocages were fabricated using a modification of a previously described protocol [13]. In brief, a PUU-POSS solution in dimethylacetamide (DMAC), with or without particulate leaching of a sodium bicarbonate (NaHCO₃) porogen, was poured onto a square-shaped glass dish mold (100 mm × 100 mm × 0.5 mm in terms of width, length and height) and coagulated by a customized TIPS process to obtain different scaffold groups (Table 1). In those cases in which the porogen was incorporated, pre-sieved NaHCO₃ (20-45 μm particle size; Sigma-Aldrich, UK) was introduced into the PUU-POSS solution prior to TIPS: PUU-POSS was mixed with Tween (Sigma-Aldrich, UK) and the NaHCO₃ particles (wt% ratio 88:2:10) in an ARE-250 planetary centrifugal mixer (Thinky Inc.; California, US) at 2,000 rpm mixing step, followed by a 1,500 rpm defoaming step. After coagulation, water was changed regularly up to 5 times over a 2-day period to watch away excess of solvent and/or NaHCO₃.

Composite collagen hydrogel functionalized cryo-coagulation scaffolds were fabricated in combination with a Real Architecture for 3D Tissues (RAFT) compression of a collagen [18] rat tail type I solution (Sigma-Aldrich, UK) infused within the scaffolds (CC and 10% NaHCO₃-CC). After RAFT absorption using hydrophilic porous absorbers (RAFT 3D systems; Lonza, UK), 10 μg/mm³ and 12 μg/mm³ of collagen per scaffold resulted respectively (CC+Collagen and 10% NaHCO₃-CC+Collagen).

Table 1 Processing conditions

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>Porogen into PUU-POSS solution</th>
<th>PUU-POSS solution poured on glass mold</th>
<th>Coagulation process</th>
<th>Thermal treatment</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room temperature coagulation + heating, (RTC+H)</td>
<td>No porogen</td>
<td>N/A</td>
<td>25°C water for 24 h</td>
<td>40°C water for 24 h</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Porogen</td>
<td>Temperature</td>
<td>Water Treatment</td>
<td>Thermal Treatment</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>-------------</td>
<td>----------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>Cryo-coagulation (CC)</td>
<td>No porogen</td>
<td>−20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
<td>No thermal treatment</td>
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</tr>
<tr>
<td>Porogen cryo-coagulation (10% NaHCO₃-CC)</td>
<td>NaHCO₃</td>
<td>−20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
<td>No thermal treatment</td>
<td></td>
</tr>
<tr>
<td>Cryo-coagulation + Collagen (CC+Collagen)</td>
<td>No porogen</td>
<td>−20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
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<tr>
<td>Porogen cryo-coagulation + Collagen (10% NaHCO₃-CC + Collagen)</td>
<td>NaHCO₃</td>
<td>−20°C for 24 h</td>
<td>0°C ice water for 24 h</td>
<td>Yes</td>
<td></td>
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</table>

2.1.2 Characterization of the scaffolds

A Zeiss Supra 35VP field emission scanning electron microscope (FE-SEM) was used to evaluate the structure and morphology of the top surface and cross-section (i.e. liquid nitrogen) of the scaffolds as produced. General descriptions were previously referred in Section 2.4 Chapter 2.

The hierarchical porous structure of the scaffolds (freeze-dried in liquid nitrogen) was evaluated with a Poremaster 60GT mercury intrusion porosimeter (Quantachrome, UK). In addition, the effect of the pore size distribution on protein adsorption was studied with a bicinchoninic acid (BCA) assay (Pierce Biotechnology, USA). Two different protein solutions were tested: bovine serum albumin (BSA) (Sigma-Aldrich, UK) and human bronchial epithelial cell medium (BEpiCM) (ScienCell, California, USA). Scaffold discs (n=6; 11 mm diameter, 0.5 mm thickness) were placed in a 48-well plate and incubated at 37°C for 24 h and up to 3 weeks with 1 mL of 500 μg/mL BSA in PBS or 1 mL of BEpiCM. The absorbance was read at 562 nm, with the protein concentration generated from a standard curve.

Dynamic stiffness softening was characterized as previously referred in Section 2.3 Chapter 2, over a 21-day period. In brief, samples of each group (n=6) at wet condition, before and after incubation at 37°C, were subjected at room temperature to an ASTM D882 standard test method for static mechanical tensile testing.

2.2 Cell culture and in vitro analysis

2.2.1 Cell selection and culture media
Detailed descriptions were previously referred in Section 5.2.2 Chapter 5. They were then clipped to CellCrown™ polycarbonate transwell housing (Figure 1) for 24-well plate (Scaffdex Oy, Tampere, Finland).

Figure 1 (A) Schematic of collagen hydrogel functionalized PUU-POSS porogen cryo-coagulated scaffold (10%NaHCO₃-CC+Collagen). (B-D) Illustration of air-liquid interface cell culture of human bronchial epithelial cells (hBEpiCs) on the scaffold: (C) monoculture and (D) co-culture conditions with either human bronchial fibroblast cells (hBFs) or human bone-marrow derived mesenchymal stem cells (hBM-MSCs) at day 1 and after 21 days.

For monoculture experiments, 1.2 mL of BEpiCM was added into each well, scaffold inserts (CC, CC+Collagen, 10%NaHCO₃-CC and 10%NaHCO₃-CC+Collagen) were placed into the wells and the upper chamber was filled with 500 μL of Pneumacult™-ALI. Scaffolds were seeded with 1.5×10⁴ cells (1.5×10⁵ cells/cm³), cultured under BEpiCM for 3 days to promote cells attachment. After day 3, the scaffolds were ‘air-lifted’ by replacing 1.2 mL of Pneumacult™-ALI to the basal chamber only, while the upper chamber was emptied to let cells exposed to air. Finally, the lower chamber was replaced with new medium every 2 days.

For co-culture experiments, 1.5×10⁴ cells (1×10⁵ cells/cm³) of either hBFs or hBM-MSCs were first seeded onto 10% NaHCO₃-CC+Collagen scaffold inserts and cultured under EME or MSCM respectively for 3 days to promote cell acclimatization and attachment. Subsequently, hBEpiCs were then seeded as described above. Samples were cultured in a 1:1 mixture of media (BEpiCM-EME or BEpiCM-MSCM).

1.8×10⁴ cells (1×10⁵ cells/cm³) were plated on to Polyethylene terephthalate (PET) inserts and cultured under Pneumacult™-ALI (STEMCELL technologies; Cambridge, UK) conditions for 3 days to promote cell attachment. After day 3, the scaffolds were ‘air-lifted’ by replacing 500 μL of Pneumacult™-ALI to the basal chamber only, while the upper chamber was left empty to allow the cells to be exposed to air.

2.2.2 Cellular proliferation and viability
Detailed descriptions were previously referred in Section 5.2.3.1 Chapter 5

2.2.3 Cellular morphology

Detailed descriptions were previously referred in Section 5.2.3.3 Chapter 5

2.2.4 Immunofluorescence staining

Detailed descriptions were previously referred in Section 5.2.3.2 Chapter 5

2.2.5 Expression of genetic markers of epithelial differentiation

Relative gene expression of epithelial cells seeded on the scaffolds was quantified using qPCR. Detailed descriptions were previously referred in Section 5.2.3.4 Chapter 5

2.2.6 Evaluation of epithelial differentiation

Enzyme-linked immunosorbent assay (ELISA) was used to detect presence of MU5AC, FOXJ1, ZO-1 and keratin 18 in scaffolds (n=4) within their cell culture medium at weeks 2 and 3. Detailed descriptions were previously referred in Section 5.2.3.5 Chapter 5

2.2.7 Evaluation of the epithelial barrier integrity

Detailed descriptions were previously referred in Section 5.2.3.7 Chapter 5

2.2.8 Evaluation of the permeability of the epithelial barrier

Detailed descriptions were previously referred in Section 5.2.3.8 Chapter 5

2.3 Data analysis

Statistical analysis of the results was performed using Graph-Pad Prism. In cases of analysis between two groups, statistical difference was assessed by the two-tailed Student t test. For multiple groups, statistical difference between groups was assessed by 1-way ANOVA at one time point or 2-way ANOVA for multiple time points, as appropriate.

3. Results and Discussion

3.1 Physico-mechanical characterization of the scaffolds

Elastomer scaffolds out of PUU-POSS were fabricated by TIPS, with or without the addition of a NaHCO₃ porogen, through micro/nano- phase separation of PUU chains and crystallization of soft segments at different thermal processing conditions (Chapter 5 Table 5.1), for which three main groups were developed: room temperature coagulation and heating (RTC+H), cryo-coagulation (CC), and porogen cryo-coagulation (10% NaHCO₃-CC).

The porosity of a scaffold and the pore size are known to have direct applications in tuning the cellular response in 3D constructs [21], and they are important features to provide appropriate nutrients and gas exchange. Scanning electron microscopy (SEM) (Figure 2 A-F) and mercury intrusion porosimetry (Figure 2 G) were used to characterize the porous structure of the scaffolds as produced. SEM imaging of the top surface confirmed a dense-skin effect for the RTC+H scaffold (Figure 2 A, D) with a limited number of visible pores. This is attributed to faster coagulation at the liquid-liquid interface between the
polymer solution and water [13] at room temperature, compared to the cryo-processing of the CC and 10% NaHCO₃-CC scaffolds. Both of these groups exhibited uniform spherulite-like bead morphology (Figure 2 B, E and C, F). Analysis with the mercury porosimeter (Figure 2 G-H, Table 1) revealed a trimodal distribution of the pores for the three scaffold groups exhibiting three main pore ranges: a predominant complex of nanopores around 5-37 nm, and two other complexes at 37-10,000 nm and 10,000-1000,000 nm. The addition of the porogen during manufacturing contributed to the high pore surface area and porosity exhibited by the 10% NaHCO₃-CC scaffold (305.49 m²/g, 96.8%) compared to CC (160.9 m²/g, 89.5%) or the RTC+H group (49.9 m²/g, 74.2%). The 10% NaHCO₃-CC sample presented the greatest porosity of all groups, and this trend was significantly higher (p<0.01) compared to both the CC and RTC+H scaffold groups (Figure 2 H). This indicates that the addition of a porogen, combined with the cryo-process during TIPS, permits achieving a hierarchical micro/nano-porous structure greater than that exhibited by cryo-coagulation alone (i.e. CC) [14]. The total porosity of the scaffolds remained unchanged even after incubation at body temperature for 21 days.

Other than porosity, protein adsorption on the scaffold has significant effects on cellular responses such as cell growth and differentiation [22]. In this regard, the effects of the pore size and pore size distribution of the various scaffolds on BSA and BEpiCM protein adsorptions were investigated (Figure 2 I-J). After 24 h, greater amount of adsorbed BSA was confirmed on the 10% NaHCO₃-CC group compared to the other two scaffold types (Figure 2 I). Significantly more BEpiCM protein was also adsorbed on 10% NaHCO₃-CC compared to PET control, RTC+H or CC scaffold groups after 24 h (p<0.05) (Figure 2 J). As expected, the BEpiCM protein adsorbed onto the scaffold continued to increase as analyzed over a period of 3 weeks (Table 3), confirming the effect of the uniform micro/nano-porous structure.

Stiffness softening was confirmed by analysing the mechanical properties of the scaffolds before and after incubation at body temperature (Figure 2 K-Q, Table 2), where the 10% NaHCO₃-CC group presented the greatest relaxation effect after 21 days, superseding that of the CC sample. The attributed thermostresponsive stiffness softening is contributed by the physical evolution of the elastomer during crystallization/melting of the soft segments and self-assembly/inverse self-assembly of the soft and hard segments of poly(urea-urethane). [13] Prior to thermal incubation, both CC and 10% NaHCO₃-CC groups exhibited significantly greater tensile mechanical properties compared to the RTC+H sample (p<0.001). While CC showed higher properties compared to those of 10% NaHCO₃-CC, the trend was non-significant in terms of the tensile modulus (at 50% strain), ultimate tensile strength and strain at break before or after incubation at body temperature. This is reminiscent of the ‘stiffness memory’ effect previously observed on these scaffolds.[13] While no significant differences between these two groups were found neither for their toughness after 21 days at 37°C, the CC group exhibited significantly (p<0.05) greater values at day 0. This data indicates that the overall mechanical properties of CC remained similar after the addition of NaHCO₃ as porogen during TIPS.
Figure 2 (A-F) SEM demonstrating variations in micro/nanostructure at (A-C) top-view and (E-F) cross-section: (A, D) RTC+H; (B, E) CC; (C, F) 10% NaHCO₃-CC. (G-H) Pore size distribution and porosity
for each conditional scaffold type (n=6) before and after 21 days incubation at 37°C. (I) BCA protein adsorption after 24 h incubation (n=6). (J) Comparison of total protein adsorbed on each conditional scaffold (n=6) with 1 mL BEpiCM medium. (K-I) Representative stress-strain curves showing stiffness softening; (N-Q) Comparison of the tensile mechanical properties of the scaffolds (n=6) before and after 21-day incubation at 37°C: (N) tensile modulus (at 50% strain), (O) ultimate tensile strength, (P) toughness, and (Q) strain at break. The differences between the experimental groups were analyzed by one-way or two-way ANOVA using Tukey’s post hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**Table 1** Pore diameter, pore volume and relative pore surface fraction of as-produced scaffolds

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Pore diameter, nm</th>
<th>Pore volume, cm³/g</th>
<th>Relative pore volume, %</th>
<th>Surface area, m²/g</th>
<th>Relative pore surface fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTC+H</td>
<td>1000,000 to 10,000</td>
<td>15.9</td>
<td>51</td>
<td>28.9</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>10,000 to 37</td>
<td>0.1</td>
<td>0.3</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>37 to 5</td>
<td>14.6</td>
<td>48.7</td>
<td>17</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>30.6</td>
<td>49.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1000,000 to 10,000</td>
<td>13.4</td>
<td>16</td>
<td>25.7</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>10,000 to 37</td>
<td>32.1</td>
<td>38</td>
<td>101.2</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>37 to 5</td>
<td>39.6</td>
<td>46</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>85.3</td>
<td>160.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% NaHCO³ - CC</td>
<td>1000,000 to 10,000</td>
<td>36</td>
<td>27.7</td>
<td>48.8</td>
<td>15.98</td>
</tr>
<tr>
<td></td>
<td>10,000 to 37</td>
<td>37</td>
<td>27.9</td>
<td>112.8</td>
<td>36.9</td>
</tr>
<tr>
<td></td>
<td>37 to 5</td>
<td>59</td>
<td>44.4</td>
<td>143.9</td>
<td>47.1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>132</td>
<td>305.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2** Physical and mechanical properties of the various scaffolds (n=6) at day 0 and after 21 days incubation at body temperature
<table>
<thead>
<tr>
<th>Scaffold/ day</th>
<th>Scaffold density, kg/m³</th>
<th>Total porosity, 100%</th>
<th>Tensile modulus, MPa</th>
<th>Ultimate strength, MPa</th>
<th>Strain at break, %</th>
<th>Toughness, J.m⁻³.10⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTC+H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D0</td>
<td>90.6 (±11.8)</td>
<td>74.2 (±7.8)</td>
<td>2.1 (±0.2)</td>
<td>2.2 (±0.2)</td>
<td>292 (±25)</td>
<td>294 (±25)</td>
</tr>
<tr>
<td>D21</td>
<td>92.1 (±9.7)</td>
<td>72.0 (±8.8)</td>
<td>2.0 (±0.4)</td>
<td>1.8 (±0.3)</td>
<td>282 (±31)</td>
<td>278 (±31)</td>
</tr>
<tr>
<td>CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D0</td>
<td>41.3 (±3.4)</td>
<td>89.4 (±13.9)</td>
<td>20.4 (±1.8)</td>
<td>20.7 (±0.4)</td>
<td>711 (±30)</td>
<td>767 (±30)</td>
</tr>
<tr>
<td>D21</td>
<td>60.3 (±3.4)</td>
<td>85.6 (±9.4)</td>
<td>8.7 (±1.7)</td>
<td>4.4 (±1.1)</td>
<td>433 (±35)</td>
<td>432 (±67)</td>
</tr>
<tr>
<td>10% NaHCO₃-CC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D0</td>
<td>28.7 (±5.4)</td>
<td>96.7 (±7.9)</td>
<td>16.8 (±1.3)</td>
<td>17.3 (±1.2)</td>
<td>767 (±32)</td>
<td>654 (±32)</td>
</tr>
<tr>
<td>D21</td>
<td>33.4 (±7.9)</td>
<td>94.3 (±7.9)</td>
<td>7.0 (±0.5)</td>
<td>3.4 (±0.3)</td>
<td>381 (±52)</td>
<td>404 (±41)</td>
</tr>
</tbody>
</table>

Table 3 Percentage of protein adsorbed on the scaffold and protein that remained in bulk solution

<table>
<thead>
<tr>
<th>Scaffold in 1 mL BEpiCM medium</th>
<th>% on material (n=5)</th>
<th>% remained in bulk solution (n=5)</th>
<th>Scaffold in 500 μg/ml BSA concentration (Total amount of BSA proteins adsorbed) n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTC+H</td>
<td>24 h</td>
<td>3.37 (±1.07)</td>
<td>96.63 (±5.55)</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>6.22 (±1.12)</td>
<td>93.78 (±8.37)</td>
</tr>
<tr>
<td>CC</td>
<td>24 h</td>
<td>12.54 (±3.49)</td>
<td>87.46 (±5.03)</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>14.11 (±4.06)</td>
<td>85.89 (±5.99)</td>
</tr>
<tr>
<td>10% NaHCO₃-CC</td>
<td>24 h</td>
<td>27.32 (±2.93)</td>
<td>72.68 (±3.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>150.32 μg/mL</td>
</tr>
</tbody>
</table>
Table 1

<table>
<thead>
<tr>
<th>Time</th>
<th>AlamarBlue Fluorescence</th>
<th>Total DNA Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 weeks</td>
<td>32.11 (±4.21)</td>
<td>67.89 (±3.75)</td>
</tr>
</tbody>
</table>

(31 ± 3.45% in 500 μg/mL BSA)

---

Figure 3 (A-B) Cellular viability and proliferation of hBEpiCs monoculture at 5×10^5 cells/cm^2 on each conditional scaffold (n=6) over a 10-day period; measured by (A) alamarBlue fluorescence assay and (B) total DNA assay. (C) Total percentage counts and staining images at day 7 for Ki67, p63 and vimentin (10 frames per scaffold type, 500 cells analyzed). (D-E) Cellular proliferation at day 7 as seen by (D) SEM and (E) confocal microscopy; cells stained for nuclei (blue), F-actin (green) and Ki67 expression (purple). (F) Immunofluorescent staining (p63, vimentin and keratin 5) indicating the organization of basal epithelial cells at day 7; maximum intensity projections of p63 (red), vimentin
(yellow) and KRT5 (blue) reconstructed from Z-stacks. Respectively for (D1-F1) hBEpiCs monoculture on CC, (D2-F2) hBEpiCs monoculture on CC+Collagen, (D3-F3) hBEpiCs monoculture on 10% NaHCO3-CC, (E4-F4) hBEpiCs monoculture on 10% NaHCO3-CC+Collagen, (D5-F5) hBEpiCs co-cultured with hBFs on 10% NaHCO3-CC+Collagen, (D6-F6) hBEpiCs co-cultured with hBM-MSCs on 10% NaHCO3-CC+Collagen. The differences between the experimental groups were analyzed by two-way ANOVA using Tukey’s post hoc test. **p<0.01; ***p<0.001; ****p<0.0001.

3.2 Epithelial cell culture on porous scaffolds

The ability of the 10% NaHCO3-CC scaffold to support viability of hBEpiCs over a 10-day period was further assessed in terms of cellular metabolic activity (Figure 3A). No significant differences were observed between the 10% NaHCO3-CC and the hybrid CC+Collagen group. Both of these trends were greater compared to the CC sample but lower to PET used as positive control. From the physico-mechanical characterization results (Figures 2), the RTC+H group presented the greatest disadvantages compared to the rest of groups, and it was clear from alamarBlue (Figure 3A) and Total DNA (Figure 3B) assays that the metabolic activity and cell proliferation reduced with time in this group. Therefore, such scaffold type was not used for further in vitro testing.

No significant differences were observed between the 10% NaHCO3-CC and the CC+Collagen groups; however, slightly greater amounts of DNA were quantified on the PET control (Figure 3B). The 10% NaHCO3-CC scaffold was therefore infused with collagen hydrogel into a hybrid scaffold (10% NaHCO3-CC+Collagen). For this case, the cellular activity reported was the greatest, with a significant trend compared to the rest of the scaffold types (p<0.001) (Figure 3A-B).

To demonstrate hBEpiCs’ cell-surface interactions with the scaffolds, SEM imaging and F-actin staining was carried out 7 days after cell seeding (Figure 3 D-E). Cells were shown to attach and spread on the material surface (Figure 3 D) in all conditions as all cells extended their lamellipodia around the edges of the cells to adhere to the material. The actin filaments were also seen protruding to the edges of the cell membranes to aid cellular attachment, movement and migration (Figure 3 E). Greater F-actin expression was seen on 10% NaHCO3-CC compared to CC or CC+Collagen (Figure 3 E) on its monoculture condition. Co-culture with hBFs or hBM-MSCs on 10% NaHCO3-CC+Collagen scaffolds superseded this effect (Figure 3 E).

Immunofluorescence against Ki67 was used to identify mitotic cells as well as basal cell markers such keratin 5 and p63 (Figure 3 F). In particular, p63 has been shown to play a critical role in the development of the normal epithelium of the airways and appears to control the commitment of early stem cells into basal cell progeny and their maintenance [23]. These underlying mechanisms include regulation of integrin receptors required for basal cell attachment and proliferation, and an antagonistic function with the Notch signalling pathway that promotes their commitment to differentiation [24]. In addition, evidence is the role of p63 in forming a stratified epithelium [24]. On the other hand, the epithelial to mesenchymal transition plays a critical role in airway remodelling [25], and vimentin is a main intermediate filament of the cytoskeleton of mesenchymal-like cells. The total percentage counts per frame and staining images at day 7 (middle stage of culture) are illustrated in Figure 3C. While slight
differences were quantified in terms of the various scaffold types, no significant differences were appreciated at this specific time point.

3.3 Epithelial mucin expression and barrier formation

The ability of the scaffolds to support hBEpiC cell differentiation was assessed by analysis of MUC5AC. MUC5AC is a gene associated with goblet cells in the respiratory epithelium, and therefore it is a substantial component of the respiratory mucus coating and an indicator of mucciliary epithelial cell differentiation [26,27]. Quantitative PCR analysis (Figure 4A) showed that MUC5AC gene expression was upregulated on hBEpiCs monoculture on both CC+Collagen and 10% NaHCO3-CC scaffolds compared to CC alone. No major differences between CC+Collagen and 10% NaHCO3-CC groups were observed, but the gene expression was lower compared to PET control. It was evident, however, that the addition collagen to the 10% NaHCO3-CC scaffold significantly (p<0.01) upregulated MUC5AC. This data indicates that the addition of collagen promotes a more sustained mucus-secreting epithelial, and highlights the positive effects of collagen as an extracellular matrix (ECM) component on epithelial cell response [28]. Indeed, a study by Davenport et al. back in 1996 had highlighted that the presence of type I collagen in the substrate induced ciliation of rat tracheal epithelial cells cultured at an ALI, something that could not be achieved on polycarbonate inserts alone [29]. Two other basement membrane components such as collagen type IV and laminin were also found to alter the basal cell attachment of oral epithelial cells and promote their differentiation into a more mucus-secreting epithelial phenotype [30], demonstrating that coatings based on natural polymers can enhance the functionality of an engineered epithelium. MUC5AC gene expression was also seen to increase when 10% NaHCO3-CC+Collagen scaffolds were co-cultured with hBFs or hBM-MSCs. Significant differences in gene expression were observed on co-cultures with hBM-MSCs compared to those with hBFs (p<0.01). Analysis of MUC5AC glycoprotein secretion to the medium corroborated these findings, where more glycoprotein presence was detected in 10% NaHCO3-CC+Collagen scaffolds compared to CC, CC+Collagen or 10% NaHCO3-CC; nevertheless, greater protein was expressed on co-cultures with hBFs or hBM-MSCs on 10% NaHCO3-CC+Collagen (Figure 4 A-B).

The ability of the scaffolds to support hBEpiC cell differentiation was further assessed by analyzing the expression of the tight junction protein ZO-1, also known as occludin-1. Similar ZO-1 gene expression (Figure 4 C) was exhibited by hBEpiC monoculture on CC+Collagen or 10% NaHCO3-CC scaffold groups. Gene expression levels of ZO-1 were significantly higher compared to CC alone (p<0.01), but lower with respect to the PET control. The greatest gene expression was exhibited by the 10% NaHCO3-CC+Collagen scaffold on either its monoculture or co-culture conditions, but significantly higher expression was presented on co-cultures with hBM-MSCs compared to PET control (p<0.0001). These results are supported by ELISA analysis of ZO-1 in the medium over a 3-week period (Figure 4 C); significantly higher ZO-1 was detected on the co-culture with hBM-MSCs compared to that with hBFs (p<0.001). Translation of ZO-1 gene into protein was also detected with immunofluorescent images capturing the presence of the tight junction protein between epithelial cells at day 21 (Figure 4 D). Furthermore, the presence of e-cadherin on hBEpiCs’ circumference (Figure 4 B) reinforces the hypothesis of the presence of an epithelial barrier on the scaffolds [31]. Indeed, their localization in the
cell periphery and their affiliation with ZO-1 protein are recognized as a core component of the epithelial barrier integrity [32].

Overall, this data demonstrates that hBEpiC cells can form a functional epithelium on the apical side of 10% NaHCO₃-CC scaffolds, with similar results to those obtained with CC+Collagen. However, better epithelial differentiation in terms of mucin phenotype and barrier formation is obtained with the addition of collagen onto 10% NaHCO₃-CC and in co-cultured conditions at an air-liquid interface.

Figure 4 (A) Comparative gene expression (n=5) in the epithelium analysis for MUC5AC as a marker protein of goblet cells of mucus production, and ELISA analysis (n=4) for presence of MUC5AC
secretion to the medium at weeks 2 and 3. (B) Immunofluorescent staining of MUC5AC glycoprotein at day 21 on the various scaffolds, respectively for hBEpiCs monoculture on CC, hBEpiCs monoculture on CC+Collagen, hBEpiCs monoculture on 10% NaHCO$_3$-CC, hBEpiCs monoculture on 10% NaHCO$_3$-CC+Collagen, hBEpiCs co-cultured with hBFs on 10% NaHCO$_3$-CC+Collagen, and hBEpiCs co-cultured with hBM-MSCs on 10% NaHCO$_3$-CC+Collagen. (C) Comparative gene expression (n=5) in the epithelium analysis for ZO-1 as a marker of tight junctions, and ELISA analysis (n=4) for presence of ZO-1 secretion to the medium at weeks 2 and 3. (D) Immunofluorescent staining of ZO-1 at day 21 on the various scaffolds. Maximum intensity projections of MUC5AC (purple) and ZO-1 (red) reconstructed from Z-stacks; cells counterstained for nuclei (blue) and e-catherin (green). The differences between the experimental groups were analyzed by two-way ANOVA using Tukey’s post hoc test. **p<0.01; ***p<0.001; ****p<0.0001.

3.4 Epithelial ciliation

Cilia are an important feature of a fully-functional tracheobronchial epithelium since they help remove debris from the airway tract [33]. In particular, FOXJ1 is a regulator of motile cilogenesis [33], and the formation of motile cilia in the scaffold cultures was initially assessed by analysing its gene expression (Figure 5A). Very limited FOXJ1 gene expression was quantified on CC scaffolds after 21 days, and while greater expression was observed on either 10% NaHCO$_3$-CC or CC+Collagen samples, greater gene expression could still be quantified on the PET sample. FOXJ1 was upregulated to a greater extent on monoculture conditions on 10% NaHCO$_3$-CC+Collagen scaffolds and in their co-culture conditions with hBFs; however, significantly higher levels were quantified when co-cultured with hBM-MSCs as compared to hBFs or monoculture on 10% NaHCO$_3$-CC+Collagen (p<0.001). Better ciliation on hBEpiCs monoculture in 10% NaHCO$_3$-CC+Collagen compared to CC or CC+Collagen was confirmed by immunofluorescence against acetylated alpha tubulin after 21 days (Figure 5B); yet, it was expressed to a greater extent on co-cultures with hBFs or hBM-MSCs on 10% NaHCO$_3$-CC+Collagen scaffolds. Further indication of such ciliation promotion on 10% NaHCO$_3$-CC+Collagen scaffolds was confirmed by ELISA analysis of FOXJ1 during a 3-week period (Figure 5A), confirming the abovementioned qPCR results.

The formation of cilia development and the expansion of a ciliated surface on the apical side of the various scaffolds were further assessed through SEM imaging (Figure 6 A-F, Table 3), with cross-sectional imaging (Figure 6G) of 10% NaHCO$_3$-CC+Collagen scaffolds confirming that an epithelial layer had covered the scaffold and that the micro/nano- porous structure was suitable for cell ciliation. The ciliary structures exhibited by hBEpiCs monoculture on 10% NaHCO$_3$-CC scaffolds were more prominent in length (1.65 ± 0.43 μm) than those observed in monoculture on CC+Collagen (1.32 ± 0.06 μm) (Figure 6H). Not a prominent ciliation could be observed on CC scaffolds (0.30 ± 0.04 μm). Higher ciliation length was exhibited by the PET film compared to the 10% NaHCO$_3$-CC scaffold (2.1 ± 0.29 vs 1.65 ± 0.43 μm respectively), and both were relatively short compared to those observed by hBEpiCs monoculture on 10% NaHCO$_3$-CC+Collagen scaffolds (4.8 ± 0.94 μm). In particular, SEM imaging revealed that cells grown on the 10% NaHCO$_3$-CC+Collagen scaffolds, either as monoculture or in co-cultured conditions, adopted a well-developed pseudostratified columnar morphology not observed in
culture with the previous scaffold types. Notably, hBEpiCs co-cultured with hBM-MSCs were longer than those observed in co-culture with hBFs (7.98 ± 1.57 vs 5.69 ± 1.09 μm). The average cilia length in human airways has been reported to be around 5 μm [34], and thus, a similar effect was provided only by the 10% NaHCO₃-CC+Collagen scaffolds. In terms of cilia diameter, non-significant differences were found among the different scaffold groups based on hBEpiCs monoculture conditions compared to PET control (Figure 6I, Table 4). However, more prominent (p<0.01) diameters were reported when hBEpiCs were co-cultured on 10% NaHCO₃-CC+Collagen scaffolds with either hBFs or hBM-MSCs, respectively being 141 ± 15 and 155 ± 23 nm. Cilia diameter in the human airway has been reported to be ~ 200 nm [35], supporting the hypothesis that a co-culture on 10% NaHCO₃-CC+Collagen scaffolds promotes better ciliation of hBEpiCs.

Table 4: Morphology and quantification of ciliation development at day 21 (n=20 counts) on the various scaffolds

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Length (μm)</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoculture PET insert</td>
<td>2.10 (±0.29)</td>
<td>92 (±11)</td>
</tr>
<tr>
<td>Monoculture on CC</td>
<td>0.30 (±0.04)</td>
<td>81 (±18)</td>
</tr>
<tr>
<td>Monoculture on CC+Collagen</td>
<td>1.32 (±0.06)</td>
<td>87 (±18)</td>
</tr>
<tr>
<td>Monoculture on 10% NaHCO₃-CC</td>
<td>1.65 (±0.43)</td>
<td>88 (±18)</td>
</tr>
<tr>
<td>Monoculture on 10% NaHCO₃-CC+Collagen</td>
<td>4.80 (±0.94)</td>
<td>110 (±17)</td>
</tr>
<tr>
<td>Co-culture with hBFs 10% NaHCO₃-CC+Collagen</td>
<td>5.69 (±1.09)</td>
<td>141 (±15)</td>
</tr>
<tr>
<td>Co-culture with hBM-MSCs on 10% NaHCO₃-CC+Collagen</td>
<td>7.98 (±1.57)</td>
<td>155 (±23)</td>
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</tbody>
</table>
Figure 5  (A) Comparative gene expression (n=5) in the epithelium analysis for FOXJ1 as a marker of proteins of ciliation, and ELISA analysis (n=4) for presence of FOXJ1 secretion to the medium at weeks 2 and 3. (B) Immunofluorescent staining of acetylated alpha tubulin as maker of ciliation at day 21 on the various scaffolds; respectively for hBEpiCs monoculture on CC, hBEpiCs monoculture on CC+Collagen, hBEpiCs monoculture on 10% NaHCO₃-CC, hBEpiCs monoculture on 10% NaHCO₃-CC+Collagen, hBEpiCs monoculture on 10% NaHCO₃-CC+Collagen (hBM-MSCs + BEpiCs).
CC+Collagen, hBEpiCs co-cultured with hBFs on 10% NaHCO₃-CC+Collagen, and hBEpiCs co-cultured with hBM-MSCs on 10% NaHCO₃-CC+Collagen. (C) Comparative gene expression (n=5) in the epithelium analysis for keratin 18 as a marker of differentiated cells, and ELISA analysis (n=4) for presence of keratin 18 secretion to the medium at weeks 2 and 3. (D) Immunofluorescent staining of intracellular keratin 14 and intracellular keratin 18 indicating the organization of basal epithelial cells and differentiated cells in the epithelium at day 21 on the various scaffolds. Maximum intensity projections of acetylated alpha tubulin (green), keratin 14 (yellow) and keratin 18 (purple) reconstructed from Z-stacks; cells counterstained for nuclei (blue). The differences between the experimental groups were analyzed by two-way ANOVA using Tukey’s post hoc test. **p<0.01; ***p<0.001; ****p<0.0001.

Figure 6 SEM images of hBEpiCs differentiation in an air-liquid interface (ALI) culture on the various scaffold types at day 21: (A) hBEpiCs monoculture on CC; (B) hBEpiCs monoculture on CC+Collagen; (C) hBEpiCs monoculture on 10% NaHCO₃-CC; (D) hBEpiCs monoculture on 10% NaHCO₃-CC+Collagen; (E) hBEpiCs co-cultured with hBFs on 10% NaHCO₃-CC+Collagen; (F) hBEpiCs co-cultured with hBM-MSCs on 10% NaHCO₃-CC+Collagen. (G) SEM of cross-sectional slices showing (G1-G2) a ciliated epithelium of hBEpiCs monoculture on 10% NaHCO₃-CC+Collagen scaffolds and
3.5 Expression of basal cell and differentiated cell markers

hBEpiCs on the various scaffolds at day 21 (Figure 5 D) were stained against keratin 14 as marker of basal cells and keratin 18 as maker of differentiated cells to distinguish the basal layer and differentiated layers within the epithelium [36]. Immunofluorescent staining showed that expression of keratin 14 was visible on the basal layer at the cell-material interface of with the scaffold, and keratin 18 was more visible at the apical upper layer of the epithelium. Presence of keratin 14 was seen on all scaffold types, but higher staining of keratin 18 was observed on hBEpiCs monoculture on 10% NaHCO₃-CC+Collagen compared to CC, CC+Collagen and 10% NaHCO₃-CC. The greatest keratin 18 expression was presented on co-cultures with hBFs or hBM-MSCs on 10% NaHCO₃-CC+Collagen scaffolds. These results suggest that more basal cells tend to be differentiated into either ciliated or secretory cells within 10% NaHCO₃-CC+Collagen scaffolds either as monoculture or co-cultured conditions, which was confirmed by Alcian blue-Periodic acid-Schiff staining and f-actin staining at day 21 of cross-sectional slices (Figure 7 A-C). Histological staining of cross-sectional samples further confirmed greater presence of ciliated and PAS positive cells on co-culture conditions on 10% NaHCO₃-CC+Collagen scaffolds.

Keratin 14-expressing cells are also a self-renewing population and progenitors for goblet and ciliated cells [37]. Analysis performed by qPCR (Figure 5 C) showed that keratin 18 gene expression was also upregulated on 10% NaHCO₃-CC+Collagen either as hBEpiCs monoculture or in co-culture with hBFs or hBM-MSCS. This data was confirmed by ELISA analysis of keratin 18 during a 3-week period (Figure 5 C).
Figure 7 (A-B) Alcian blue - Periodic acid-Schiff (AB-PAS) staining and F-actin staining at day 21 of cross-sectional slices showing well-developed ciliated cells and mucous substance (magenta at arrow head) on the scaffolds as in respiratory mucosa: (A1-B1) hBEpiCs monoculture on CC; (A2-B2) hBEpiCs monoculture on CC+Collagen; (A3-B3) hBEpiCs monoculture on 10% NaHCO3-CC; (A4-B4) hBEpiCs monoculture on 10% NaHCO3-CC+Collagen; (A5-B5) hBEpiCs co-cultured with hBFs on 10% NaHCO3-CC+Collagen; (A6-B6) hBEpiCs co-cultured with hBM-MSCs on 10% NaHCO3-CC+Collagen. (C) Total percentage of cell count as seen by AB-PAS staining (10 frames per scaffold type, 500 cells analyzed). The differences between the experimental groups were analyzed by one-way or two-way ANOVA using Tukey’s post hoc test. * p<0.05; ****p<0.0001.
3.6 Epithelial barrier integrity

The integrity of the differentiated epithelial layers on the various scaffold groups was further quantified by means of a TEER (Figure 8 A) technique, used to indirectly quantify tight junction formation [38]. After a plateau was reached (Figure 8 B), hBEpiCs cultured on the various scaffolds formed a barrier that was >450 Ωcm², with mean TEER values at day 14 of 489 (±183), 707 (±294), 698 (±139) and 729 (±122) Ωcm² for monoculture on CC, CC+Collagen, 10% NaHCO₃-CC and 10% NaHCO₃-CC+Collagen (Table 5). It is evident that hBEpiCs cultured on the 10% NaHCO₃-CC and 10% NaHCO₃-CC+Collagen scaffolds displayed greater TEER values, relating to increased tight junction formation [38]. The co-culture with hBFs or hBM-MSCs further increased the TEER values up to 910 (±189) and 970 (±311) Ωcm² respectively. TEER values from ex vivo human lung tissue have not been reported so far, but they have been shown to exhibit a range of 300 to 650 Ωcm² on rabbit tissue and human primary epithelial cell cultures [39]. The TEER value measured for the PET control was of 460 (±79) Ωcm², which is lower than the reported data [39].

Figure 8 (A) Transepithelial electrical resistance (TEER) over a 14-day period (n=36 measurements). (B) Average TEER values of epithelial cell barriers following plateau of electrical resistance (≥day 14). (F) Apparent permeability coefficient (Papp) of fluorescein isothiocyanate-labelled dextran 70 (FITC-dextran) through the bronchial epithelial cell barrier at day 14 (n=36 measurements).

Table 5 Transepithelial electrical resistance (TEER) of the various scaffolds (n=36 measurements)
<table>
<thead>
<tr>
<th>Scaffold</th>
<th>TEER (Ωcm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Monoculture PET insert</td>
<td>90 (±37)</td>
</tr>
<tr>
<td>Monoculture on CC</td>
<td>230 (±35)</td>
</tr>
<tr>
<td>Monoculture on CC+Collagen</td>
<td>240 (±48)</td>
</tr>
<tr>
<td>Monoculture on 10% NaHCO₃-CC</td>
<td>290 (±31)</td>
</tr>
<tr>
<td>Monoculture on 10% NaHCO₃-CC+Collagen</td>
<td>490 (±31)</td>
</tr>
<tr>
<td>Co-culture with hBFs on 10% NaHCO₃-CC+Collagen</td>
<td>321 (±33)</td>
</tr>
<tr>
<td>Co-culture with hBM-MSCs on 10% NaHCO₃-CC+Collagen</td>
<td>498 (±198)</td>
</tr>
</tbody>
</table>

Table 6 Apparent permeability coefficient at day 14 on the various scaffolds (n=36 measurements)

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Papp (cm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoculture PET insert</td>
<td>5×10⁻⁴ (±1.1×10⁻⁴)</td>
</tr>
<tr>
<td>Monoculture on CC</td>
<td>4×10⁻⁴ (±1.4×10⁻⁴)</td>
</tr>
<tr>
<td>Monoculture on CC+Collagen</td>
<td>1.1×10⁻⁴ (±0.4×10⁻⁴)</td>
</tr>
<tr>
<td>Monoculture on 10% NaHCO₃-CC</td>
<td>0.9×10⁻⁴ (±0.3×10⁻⁴)</td>
</tr>
<tr>
<td>Monoculture on 10% NaHCO₃-CC+Collagen</td>
<td>0.5×10⁻⁴ (±0.1×10⁻⁴)</td>
</tr>
<tr>
<td>Co-culture with hBFs 10% NaHCO₃-CC+Collagen</td>
<td>0.3×10⁻⁴ (±0.1×10⁻⁴)</td>
</tr>
<tr>
<td>Co-culture with hBM-MSCs on 10% NaHCO₃-CC+Collagen</td>
<td>0.2×10⁻⁴ (±0.5×10⁻⁵)</td>
</tr>
</tbody>
</table>

In addition, paracellular transport of FD70 was studied in the various scaffold groups (Figure 8 C, Table 6). The Papp values obtained agree with the abovementioned TEER measurements, with the 10% NaHCO₃+CC+Collagen scaffolds, either on its monoculture or co-culture conditions, being less
permeable to solute flux compared to those cells culture on the other scaffold types. This can be explained in terms of the predicted pore radii of the cell layers [19] on the 10% NaHCO₃+CC+Collagen scaffold, where it is assumed that the lower the apparent permeability the smaller the pore radii of the cell layers [40]. In particular, the predicted pore radii for monoculture conditions were 54 nm, 26 nm, 25 nm and 16 nm for CC, CC+Collagen, 10% NaHCO₃-CC and 10% NaHCO₃-CC+Collagen scaffolds, and 8.9 nm and 7.2 nm in co-culture conditions with hBFs and hBM-MSCs respectively on 10% NaHCO₃-CC+Collagen scaffolds. Overall, these results indicate that the 10% NaHCO₃+CC+Collagen scaffold can display a more functional epithelial barrier.

4. Conclusion

This study demonstrates that the addition of a porogen-leaching salt during manufacturing of a nanocomposite polyurethane scaffold based on a TIPS technique can maintain epithelium differentiation of basal cells towards cilia and goblet cells, which are characteristic of a functional tracheobronchial epithelium. Similar physico-mechanical properties were exhibited by porogen cryo-coagulated scaffolds compared to cryo-coagulation alone, and a comparable epithelium was obtained with respect to that of hybrid collagen cryo-coagulated scaffolds. This demonstrates the feasibility of introducing a porogen during the manufacturing process to reduce associated costs related to the use of collagen. Nevertheless, a more developed ciliation and a more mucin secretion phenotype was obtained on PET films used as control. The addition of collagen to make hybrid porogen cryo-coagulated scaffolds considerably improved the functionality of the construct on its monoculture condition, but co-culture with either hBFs or hBM-MSCs still generated a more physiologically relevant model of the respiratory epithelium. Overall, this data indicates that the presence of a natural ECM component in the scaffold, as well as that of a co-culture system and increased porosity of the construct, are all elements beneficial to establish improved differentiation of the tracheobronchial epithelium.
References


Appendix. F

3D-printed elastomer for a bronchial epithelial joint cartilage analogue of the upper respiratory airway

1. Introduction
Gaining an understanding of the airway injury and repair mechanism is a main focus in the field of respiratory medicine [1]. The tracheobronchial epithelium is of particular interest; people suffering from a dysfunctional tracheobronchial epithelium results in a delayed regeneration of the epithelium with increasing risk of infection. Indeed, airway diseases represent a leading cause of death in the world.

Three main cell populations are found in the tracheobronchial epithelium: basal, ciliated, and goblet cells. Basal cells act as a stem cell population at the epithelial basement membrane during growth and tracheal injury [2,3]; ciliated cells expel inhaled particles [4,5] and goblet cells produce mucus and immunoprotective proteins used as defense mechanisms [6]. However, despite the key role of the tracheobronchial epithelium against many lung diseases, the underlying developmental biology is not fully understood. While the cross-talk between the mesenchyme and epithelium of the tracheal tissue has not been researched extensively, recent studies have reported that mesenchymal cells underlying the tracheal epithelium, the place where maturation of tracheal chondrocytes occur [7,8], pinpoint signaling pathways essential for development and repair of the trachea [9]. It has been shown that tracheal cartilage has the ability to signal to the adjacent epithelium to induce proper epithelial cell differentiation [7,10,11]. Indeed, a lack of proper cartilage affects mRNA expression of many epithelial differentiation markers [7,8].

Nanohybrid soft scaffolds from silsesquioxane elastomers with tunable hierarchical porous structures were recently fabricated by reverse 3D printing in combination with thermally-induced phase separation (TIPS) [12]. Phase transition at body temperature from the semicrystalline phase to the rubber phase, and reverse self-assembling of the quasi-random nanophase structure of the nanohybrid, have been systematically characterized to prove the role of dynamic thermoresponsive stiffness softening in regulating the proliferation of human dermal fibroblasts and differentiation of mesenchymal stem cells in vitro [12–14], tissue ingrowth, vascularization and macrophage polarization in vivo [15].

Dynamic thermoresponsive stiffness 3D-TIPS scaffolds are demonstrated to allow the co-culture of human bronchial epithelial cells (hBEpiCs) with human bone-marrow derived mesenchymal stem cells (hBM-MSCs). The co-culture tracheobronchial epithelial tissue analogue based on 3D-TIPS is further modified by engineering a joint epithelial-cartilage joint to develop an enhanced representative model that can serve as a future screening platform for oral drug discovery or therapeutic testing, and tissue regeneration.

2. Materials and Methods
2.1 Scaffold fabrication
Elastomer nanohybrid 3D-TIPS scaffolds from a polyhedral oligomeric silsesquioxane terminated poly(urea-urethane) polymer solution (PUU-POSS) were fabricated in Section 3.2.1 Chapter 3
2.2 Cell culture

2.2.1 Cell selection and culture media

General descriptions were previously referred in Section 5.2.2.1 Chapter 5.

2.2.2 Seeding cells on 3D-TIPS transwell inserts

General descriptions were previously referred in Section 5.2.2.2 Chapter 5, and Figure 1.

![Diagram](image)

**Figure 1** Schematic representation of 3D-TIPS scaffolds during cell culture: (A) 3D-TIPS scaffolds prior to seeding; (B) Cell culturing of hBEpiCs in monoculture conditions at day 1 and at an air-liquid interface after 21 days; (B1) Co-culture of hBEpiCs with hBM-MSCs at day 1 and at an air-liquid interface after 21 days; (C2) Co-culture of hBEpiCs with chondrogenic differentiated hBM-MSCs at day 1 and at an air-liquid interface after 21.

2.3 In vitro analysis

2.3.1 Histology

General descriptions were previously referred in Section 5.2.3.6 Chapter 5. Alcian blue (A-Blue) for polysaccharide indication (e.g. glycosaminoglycans), antibody collagen II (COL2) and antibody SOX9 (Sox-9) staining for collagen II and SOX9 production to indicate chondrogenic differentiated hBM-MSCs. Images were taken using a digital slide scanner (Leica SCN400F, Germany).

2.3.2 Production of sulfated glycosaminoglycans (sGAG)

The amount of sGAG (n=6) was quantified after 21 days with a Blyscan™ sulphated glycosaminoglycan assay (Biocolor Ltd.; Antrim, UK), normalized to total DNA levels. Briefly, the absorbance of dye-bound
sGAG removed by centrifugation and resuspended in dissociation reagent was read at 630 nm using a microplate reader (Biotek; Swindon, UK), calculated using a standard curve obtained from glycosaminoglycan standards provided with the kit.

### 2.3.4 Immunofluorescence
General descriptions were previously referred in Section 5.2.3.2 Chapter 5

### 2.3.5 Scanning electron microscopy (SEM)
General descriptions were previously referred in Section 5.2.3.3 Chapter 5

### 2.3.6 Quantitative reverse-transcriptase polymerase chain reaction (qPCR)
Relative gene expression of epithelial cells seeded on the scaffolds was quantified using qPCR as previously described in Section 5.2.3.4 Chapter 5

### 2.3.7 ELISA analysis
Chondrogenic presence of glycosaminoglycans, Collagen II and Aggrecan was detected within their cell culture medium at week 3 (n=4) using an enzyme-linked immunosorbent (ELISA) assay. ELISA kits used were human COL2 ELISA kit (Abbexa; Cambridge UK), human GAGs ELISA kit (Abbexa; Cambridge UK) and human Aggrecan ELISA (Abcam, UK). General descriptions were previously referred in Section 5.2.3.5 Chapter 5

### 2.3.8 Transepithelial electrical resistance (TEER) measurement
General descriptions were previously referred in Section 5.2.3.7 Chapter 5

### 2.3.9 Fluorescein isothiocyanate (FITC)-labeled dextran 70 (FD70) permeability assay
General descriptions were previously referred in Section 5.2.3.8 Chapter 5

### 2.4 Data analysis
Statistical analysis of the results was performed using Graph-Pad Prism. For multiple groups, statistical difference was assessed by one-way ANOVA at one time point or two-way ANOVA for multiple time points, as appropriate. Tukey’s post hoc analysis was performed in all ANOVA assessments.

### 3. Results

#### 3.1 Chondrogenic differentiation of hBM-MSCs
Effective differentiation of hBM-MSCs towards the chondrogenic lineage was evaluated on 3D-TIPS scaffolds engineered with a cartilage layer. Chondrogenic differentiation in the joint cartilage-epithelial 3D-TIPS analogue scaffold was evident from the bottom flat-sectional slices at day 21 stained against H&E, A-Blue, Collagen II and Sox-9 compared to the compared to the 3D-TIPS control in which hBEpiCs were co-cultured with non-differentiated hBM-MSCs (Figure 2 A-B). Immunofluorescent staining at week 3 revealed the presence of Aggrecan only on the 3D-TIPS joint analogue, as well as more prominent Collagen II formation (Figure 2 C-D). Sulfated glycosaminoglycans (sGAGs) are an effective marker of chondrogenic differentiation, and these were produced as well to a higher extent (p<0.0001) (Figure 2 E). In addition, comparative expression of chondrogenic genes expressing Collagen II, SOX9 and Aggrecan were significantly upregulated (p<0.0001) on such scaffold group (Figure 2 F). These results were further confirmed by quantification of glycosaminoglycans, Aggrecan and Collagen II
secreted to the medium (p<0.0001) at week 3 by ELISA (Figure 2 G).

Figure 2 Chondrogenic presence in a joint cartilage-epithelial analogue of hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs compared with hBEpiCs co-cultured with non-differentiated hBM-MSCs on 3D-TIPS. (A-B) H&E, A-Blue, SOX-9 and Collagen II staining at day 21 of bottom flat-sectional slices. (C-D) Immunofluorescent staining at day 21; cells stained for nuclei (red), Aggrecan (green) and Collagen II (blue). (E) Production of sulfated glycosaminoglycan (n=6) after 21 days. (F) Comparative gene expression (n=5) at day 21 in the analysis of chondrogenic differentiation for Collagen II, SOX9 and Aggrecan expression. (G) ELISA analysis (n=4) for presence of glycosaminoglycans, Collagen II and Aggrecan released to the medium at day 21. The differences between the experimental
groups were analyzed by one-way ANOVA using Tukey's post hoc test. ****p<0.0001.

3.2 Epithelial cell culture on the scaffolds

Immunofluorescent staining against keratin 5 as a basal cell marker, vimentin as a main intermediate filament of the cytoskeleton of mesenchymal-like cells, and Ki67 as a proliferative maker, were assessed at day 3 on hBEpiCs seeded on the 3D-TIPS scaffolds either on monoculture or co-culture conditions (Figure 3 A-C), along with the total percentage count per frame (Figure 3 D). Similar expression levels of all markers were quantified on the various 3D-TIPS scaffold groups.
Figure 3 Cellular proliferation of hBEpiCs at day 3 on each conditional scaffold as seen by confocal microscopy and SEM: (A) hBEpiCs monoculture on TIPS; (B) hBEpiCs co-cultured with hBM-MSCs on 3D-TIPS; (C) hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs on 3D-TIPS. Cells were stained for nuclei (blue), F-actin (green), keratin 5 (red), vimentin (yellow) and Ki67 expression (purple). (D) Count percentage of cells/mm² expressing Ki67, vimentin and keratin 5 on the scaffolds (10 frames)
per scaffold type, 500 cells analyzed). The differences between the experimental groups were analyzed by one-way ANOVA using Tukey’s post hoc test; n.s non-significant

3.3 Epithelial ciliation

The expansion of a ciliated surface on the apical side of the scaffolds was assessed through SEM imaging (Figure 4 A-D). Cells grown on the 3D-TIPS scaffolds adopted a well-developed pseudostratified columnar morphology. In particular, the ciliary structures exhibited by hBEpiCs co-cultured on 3D-TIPS scaffolds (Figure 4 C-D) were more prominent in length (p<0.001) than those observed in monoculture on PET (Figure 4 A). Non-significant differences in terms of cilia diameter or cilia length (Figure 4 E-F) were observed in the 3D-TIPS cartilage joint epithelium analogue compared to the 3D-TIPS co-culture control.

The formation of cilia development on the scaffolds was also assessed by analyzing the expression of FOXJ1, a master regular of cilogenesis, by qPCR and ELISA analysis (Figure 5 E-F). An upregulation of FOXJ1 gene expression was exhibited by 3D-TIPS compared to the PET cell insert control, and significant differences (p<0.0001) were observed compared to the control 3D-TIPS scaffold. The presence of FOXJ1 translated into protein and released to the medium was further quantified by ELISA at week 3, confirming significantly greater presence (p<0.0001) on cells cultured under the presence of a cartilage layer.
Figure 4 SEM images of hBEpiCs differentiation in an air-liquid interface (ALI) culture on the scaffolds. (A-D) Well-developed cilia and expansion of ciliated epithelial surface were observed at week 3 under SEM: (A) hBEpiCs monoculture on PET; (B) hBEpiCs monoculture on TIPS; (C) hBEpiCs co-cultured with hBM-MSCs on 3D-TIPS; (D) hBEpiCs co-culture with chondrogenic differentiated hBM-MSCs on 3D-TIPS. (E) Average (n=20 counts) mean cilia length and (F) average mean cilia diameter (n=20 counts) as seen by SEM. The differences between the experimental groups were analyzed by two-way ANOVA using Tukey’s post hoc test, or two-tailed unpaired Student's t test; ***p<0.001.
Table 1 Morphology and quantification of ciliation development at day 21 (n=20 counts) on the scaffolds

<table>
<thead>
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<th>Scaffold</th>
<th>Length (μm)</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoculture PET insert</td>
<td>2.1 (±0.29)</td>
<td>92 (±10)</td>
</tr>
<tr>
<td>Co-culture with hBM-MSCs on 3D-TIPS</td>
<td>5.98 (±1.09)</td>
<td>102 (±17)</td>
</tr>
<tr>
<td>Co-culture with chondrogenic differentiated hBM-MSCs on 3D-TIPS</td>
<td>7.98 (±1.57)</td>
<td>127 (±18)</td>
</tr>
</tbody>
</table>

3.4 Epithelial mucin expression and barrier formation

The ability of hBEpiC cells on the scaffolds to differentiate and develop a functional epithelium barrier was assessed by analyzing gene expression of the tight junction protein ZO-1 (Figure 5 E). hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs on 3D-TIPS exhibited a significant upregulation (p<0.01) compared to the 3D-TIPS control. Translation of ZO-1 gene into protein was detected by immunohistochemistry (Figure 5 A1-D1), with immunofluorescent images capturing the presence of the tight junction protein between epithelial cells at day 21 to a greater extent on the fabricated jointed analogue. ELISA analysis of ZO-1 (p<0.001) translated into protein and released into the medium at week 3 corroborated this data (Figure 5 F). Overall, these results indicate that adjacent epithelial cells formed more intercellular junctions with hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs on 3D-TIPS scaffolds than when they were co-cultured with non-differentiated hBM-MSCs.

The ability of the scaffolds to support mucin secretion was also evaluated in terms of analysis expression of MUC5AC, which is associated with goblet cells in the respiratory epithelium. Results obtained with qPCR (Figure 5 E) showed that more (p<0.01) MUC5AC gene is expressed on 3D-TIPS scaffolds with hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs compared to the 3D-TIPS control. As seen from immunofluorescent imaging at day 21 (Figure 5 A4-D4), more MUC5AC glycoprotein was expressed with chondrogenic differentiated hBM-MSCs, which was confirmed by quantification (p<0.01) of release protein to the medium by ELISA (Figure 5 F). This data indicates that hBEpiCs co-cultured at an ALI with chondrogenic differentiated hBM-MSCs on 3D-TIPS scaffolds can promote a sustained mucus-secreting epithelial phenotype that does not occur under the same co-culture conditions with non-differentiated hBM-MSCs.

3.5 Expression of differentiated epithelial cells

Comparative gene expression of keratin 18 as marker of differentiated epithelial cells was carried out at week 3 (Figure 5 E), along with protein quantification released to the medium by ELISA (Figure 5 F). Significant differences between the 3D-TIPS scaffold groups were appreciated, with greater presence (p<0.01) of keratin 18 on hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs on 3D-TIPS scaffolds. This was corroborated qualitatively by immunofluorescent staining at day 21 (Figure 5 A3-D3).

Histological examination at day 21 of cross-sectional cell-laden scaffolds showed well-developed ciliation and mucosubstance by Alcian Blue (A-Blue) and Alcian Blue-Periodic acid-Schiff (AB-PAS)
staining (Figure 6 A-B). 3D-TIPS scaffolds with an engineered cartilage layered exhibited a more prominent upper ciliated monolayer (Figure 6 B), and confocal microscopy images at day 21 qualitatively demonstrated greater presence of acetylated alpha tubulin and MUC5AC stained cells on this scaffold group (Figure 6 D) compared to control (Figure 6 C). Greater number of ciliated cells (p<0.001) were quantified on this scaffold group, and less number of non-ciliated cells (p<0.01) on the 3D-TIPS control scaffold (Figure 6 E)

Figure 5 (A-D) Immunofluorescent staining at day 21 by confocal microscope. Cells stained for nuclei
(light blue), MUC5AC (yellow), ZO-1 (red), SOX9 (green), keratin 5 (purple) and Collagen II (dark blue): (A) hBEpiCs monoculture on PET; (B) hBEpiCs monoculture on 3D-TIPS; (C) hBEpiCs co-cultured with hBM-MSCs on 3D-TIPS; (D) hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs on 3D-TIPS. (E) Comparative gene expression (n=5) at day 21 in the epithelium analysis for ZO-1, MUC5AC, FOXJ-1 and keratin 18 as markers for tight junctions, mucin expression, ciliation and epithelial differentiated cells. (F) ELISA analysis (n=4) for presence of ZO-1, MUC5AC, FOXJ-1 and keratin 18 released to the medium at day 21. The differences between the experimental groups were analyzed by one-way ANOVA using Tukey's post hoc test. **p<0.01; ***p<0.001; ****p<0.0001.
Figure 6 (A-B) Hematoxylin & Eosin (H&E), Alcian blue (A-Blue) and Alcian Blue- Periodic acid-Schiff (AB-PAS) staining at day 21 of cross-sectional slices showing gross cell morphology distribution, developed ciliation and mucosubstance formation. (C-D) Confocal microscopy images at day 21 showing e-cadherin (green), acetylated alpha tubulin (red), and MUC5AC (purple); cell nuclei counterstained in blue. (A, C) hBEpiCs co-cultured with non-differentiated hBM-MSCs on 3D-TIPS; (B, D) hBEpiCs co-culture with chondrogenic differentiated hBM-MSCs on 3D-TIPS. (E) Ciliated, non-ciliated and PAS-positive count percentage of cells/mm² (10 frames per scaffold type, 500 total cells analyzed). The differences between the experimental groups were analyzed by one-way ANOVA using Tukey’s post hoc test; **p<0.01; ***p<0.001.

3.6 Epithelial barrier integrity

The barrier integrity and functionality of the epithelium on the scaffolds was quantified by measurement of TEER over a 14-day period (Figure 7 A-B, Table 2). In both 3D-TIPS co-culture groups, hBEpiC cells formed a barrier that was >450 Ωcm², with mean TEER values at day 14 of 1187 (±232) and 1110 (±315) Ωcm² (non-significant difference), respectively, for the 3D-TIPS control and the 3D-TIPS cartilage-epithelial joint analogue.

The ability of the epithelial barrier to impede paracellular transport was also assessed in the scaffolds (Figure 7 C). hBEpiC cells co-cultured at an ALI with hBM-MSC were less permeable to solute flux than cells co-cultured with chondrogenic differentiated hBM-MSCs (non-significant difference) (Table 3), with a predicted pore radius of the cell layers of 11.3 and 9.7 nm respectively.

Figure 7 Transepithelial electrical resistance (TEER) of hBEpiCs cultured in co-culture conditions with
hBM-MSCs or chondrogenic differentiated hBM-MSCs for 14 days at an air-liquid interface on 0.385 cm² cell culture supports. (a) TEER over a 14-day course (n=36 measurements). (b) Average TEER values (n=36 measurements) of epithelial cell barriers following plateau of electrical resistance (≥ day 14). (c) Apparent permeability coefficient (Papp) of fluorescein isothiocyanate-labelled dextran 70 (FITC-dextran) through the bronchial epithelial cell barrier at day 14 (n=36 measurements). The differences between the experimental groups were analyzed by one-way or two-way ANOVA using Tukey’s post hoc test. **** p<0.0001; *** p<0.001; n.s non-significant.

<table>
<thead>
<tr>
<th>Day</th>
<th>Empty epithelial insert on 3D-TIPS</th>
<th>Co-culture with hBM-MSCs on 3D-TIPS</th>
<th>Co-culture with chondrogenic differentiated hBM-MSCs on 3D-TIPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>90 (±7)</td>
<td>312 (±67)</td>
<td>362 (±197)</td>
</tr>
<tr>
<td>5</td>
<td>140 (±19)</td>
<td>659 (±211)</td>
<td>281 (±149)</td>
</tr>
<tr>
<td>7</td>
<td>180 (±4)</td>
<td>1279 (±487)</td>
<td>1320 (±243)</td>
</tr>
<tr>
<td>10</td>
<td>80 (±15)</td>
<td>1372 (±322)</td>
<td>1425 (±489)</td>
</tr>
<tr>
<td>14</td>
<td>170 (±30)</td>
<td>1187 (±232)</td>
<td>1110 (±315)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Papp (cm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty epithelial insert on 3D-TIPS</td>
<td>590x10⁻⁶ (±42 x10⁻⁶)</td>
</tr>
<tr>
<td>Co-culture with hBM-MSCs on 3D-TIPS</td>
<td>25x10⁻⁶ (±3 x10⁻⁶)</td>
</tr>
<tr>
<td>Co-culture with chondrogenic differentiated hBM-MSCs on 3D-TIPS</td>
<td>36x10⁻⁶ (±4 x10⁻⁶)</td>
</tr>
</tbody>
</table>

4. Discussion
The objective of this study was to assess the functionality of a tracheobronchial in vitro epithelial co-culture model based on a bronchial epithelial joint cartilage analogue. It was sought here to use a 3D printed elastomer nanohybrid scaffold as the primary substrate to develop a cartilage layer via chondrogenic differentiation of hBM-MSCs to which a human epithelial cell line was seeded on top.

Co-culture systems in 2D are an established method to improve the differentiation of the respiratory
epithelium, and their translation into 3D structures [16,19,20] have paved the way into generating more accurately *in vitro* models [21]. In most of these cases, however, a microenvironment with a truly resemblance of the native tracheal tissue is difficult to replicate *in vitro*. While the cross-talk between the tracheal mesenchyme and the tracheal epithelium has not been researched extensively, it has been recently reported that cartilage has a significant role in the modulation of differentiation and metabolism, as well as in the expression of inflammatory-related genes in the tracheal epithelium [7,10,11,22,23]. To this regard, a 3D printed cartilage analogue based on chondrogenic differentiated hBM-MSCs was evaluated for its ability to allow hBEpiCs cell growth and differentiation, compared with a similarly developed 3D printed membrane in which hBEpiCs were co-cultured with non-differentiated hBM-MSCs.

Chondrogenic differentiation of hBM-MSCs on the 3D printed joint analogue was confirmed by histology, immunohistochemistry, qPCR and ELISA analysis (*Figure 2*) after 3 weeks of co-culture. Indeed, chondrogenic markers [24] such as SOX9, Aggrecan, Collagen II and synthesis of glycosaminoglycans were significantly upregulated on these scaffolds compared to the 3D-TIPS control.

The scaffold was also assessed in terms of a functional epithelium. In particular, mucin secretion is an important function of the airway tract to trap particulate matter and external pathogens. MUC5AC is an indicator of mucociliary epithelial cell differentiation [25,26], and the presence of a cartilage layer on the 3D-TIPS scaffold was able to directly increase (p<0.01) gene expression of MUC5AC compared to the control 3D-TIPS (*Figure 5 E*). Significantly (p<0.01) more MUC5AC protein was also released to the medium as analyzed by ELISA (*Figure 5 F*).

Ciliation is also an important feature of a fully-functional tracheobronchial epithelium since cilia help remove particulates and debris from the airway [28]. Development of cilia in the co-culture systems was evaluated in terms of FOXJ1 gene expression, a master regulator of motile cilogenesis [28]. Significant differences between the two 3D-printed scaffold groups were observed in terms of their gene expression (*Figure 5 E*) and their translation into protein as seen by ELISA (*Figure 5 F*), suggesting that the presence of chondrogenic differentiated hBM-MSCs during co-culture with a human epithelial cell line is beneficial into developing a more appropriate epithelium. SEM imaging was also carried out to observe and quantify the morphology of the microvilli (*Figure 4 A-D*). While microvilli of hBEpiCs co-cultured with chondrogenic differentiated hBM-MSCs were found to be larger in length (7 (±1) μm) and diameter (127 (±18) nm) compared to those exhibited by hBEpiCs co-cultured with non-differentiated hBM-MSCs (respectively 5 (±1) μm and 102 (±17) nm), these were non-significant between the two groups. The average cilia length and average cilia diameter in human airways has been reported to be around 5 μm [29], and 200 nm respectively [30].
Epithelial barrier integrity was evaluated in terms of TEER. Quantitative barrier analysis using TEER (Figure 7 A-B) showed TEER values of 1187 (±232) and 1110 (±315) Ωcm² respectively for hBEpiCs co-cultured at an ALI with hBM-MSCs or with chondrogenic differentiated hBM-MSCs. This trend was non-significant between the two groups, though. These results indicate that either scaffold group contribute equally to increase the tight junction formation [31] as seen by TEER compared to that of PET used as control of epithelial differentiation. TEER values from ex vivo human lung tissue have not been reported so far, but it has been shown to exhibit a range of 300 to 650 Ωcm² on rabbit tissue and human primary epithelial cell cultures [32]. Analysis of paracellular permeability using FD70 confirmed that the epithelial barrier formed on the 3D-TIPS scaffolds, as a co-culture of hBEpiCs with either hBM-MSCS or chondrogenic differentiated hBM-MSCs, was significantly less permeable (p<0.0001) to solute flux compared to the PET control. While the predicted pore radii of the epithelial cell layer was found smaller for the 3D-TIPS control, non-significant differences were found with respect to the 3D-TIPS epithelial-cartilage joint analogue (Figure 7 C). This can be explained in terms of a higher predicted pore radii of the cell layers [17,33] in the former group, but the flux was still smaller compared to previously reported Papp values [34].

Overall, the results presented here justify the hypothesis that the presence of a cartilage layer during co-culture with a human epithelial cell line can facilitate enhanced expression of markers typical of a functional tracheobronchial epithelial model [7,10,11].

5. Conclusions
This study has developed a 3D printed joint cartilage-epithelial analogue as a 3D in vitro airway model. The scaffold presented here combines a cartilage layer from chondrogenic-differentiated hBM-MSCs, used as a primary substrate to be co-cultured with a human epithelial cell line seeded on top. The joint tissue analogue demonstrated the ability to enhance differentiation and development of human bronchial epithelium compared to the use of non-differentiated hBM-MSCs in similar co-culture conditions. A 3D printing approach can be used as a customizable reproducible technology to generate a physiologically relevant 3D system to advance our understanding of airway disease and subsequent drug discovery.

References


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doi:10.1371/journal.pone.0008157.


