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# The development of a tissue-engineered tracheobronchial epithelial model using a bilayered collagen-hyaluronate scaffold

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ABSTRACT

Today, chronic respiratory disease is one of the leading causes of mortality globally. Epithelial dysfunction can play a central role in its pathophysiology. The development of physiologically-representative *in vitro* model systems using tissue-engineered constructs might improve our understanding of epithelial tissue and disease. This study sought to engineer a bilayered collagen-hyaluronate (CHyA-B) scaffold for the development of a physiologically-representative 3D *in vitro* tracheobronchial epithelial co-culture model. CHyA-B scaffolds were fabricated by integrating a thin film top-layer into a porous sub-layer with lyophilisation. The film layer firmly connected to the sub-layer with delamination occurring at stresses of 12–15 kPa. Crosslinked scaffolds had a compressive modulus of 1.9 kPa and mean pore diameters of 70 μm and 80 μm, depending on the freezing temperature. Histological analysis showed that the Calu-3 bronchial epithelial cell line attached and grew on CHyA-B with adoption of an epithelial monolayer on the film layer. Immunofluorescence and qRT-PCR studies demonstrated that the CHyA-B scaffolds facilitated Calu-3 cell differentiation, with enhanced mucin expression, increased ciliation and the formation of intercellular tight junctions. Co-culture of Calu-3 cells with Wi38 lung fibroblasts was achieved on the scaffold to create a submucosal tissue analogue of the upper respiratory tract, validating CHyA-B as a platform to support co-culture and cellular organisation reminiscent of *in vivo* tissue architecture. In summary, this study has demonstrated that CHyA-B is a promising tool for the development of novel 3D tracheobronchial co-culture *in vitro* models with the potential to unravel new pathways in drug discovery and drug delivery.

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## 1. Introduction

Today, chronic respiratory disease is one of the leading causes of mortality globally. Airway diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) have been identified as the fifth highest cause of mortality worldwide and are estimated to rise to fourth place by 2030 [1]. Additionally, tracheal, bronchial, and other lung cancers are predicted to become the sixth leading cause of mortality by the same year. At the core of many of these debilitating conditions, epithelial dysfunction can play a central role in their pathophysiology. For example, cystic fibrosis is rooted in defective ion transport across epithelia that drives bacterial colonisation and inflammation in the lungs [2]. The epithelium in the respiratory tract is of critical importance for the maintenance of homeostasis with key roles in lining the airways for protection, in mediating interaction with the external environment, and in regulating innate immune responses [3,4]. Accordingly,

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effective drug targeting of epithelial tissue and restoration of its normal function is a significant factor in the design of therapeutics and treatment of such diseases. Furthermore, the airways are a very attractive route for systemic drug delivery to treat a range of non-respiratory diseases, with a large absorptive surface area, thin alveolar epithelium barrier, high blood flow and relatively low drug-degrading metabolic activity. Indeed, this route is of great interest for delivery of complex biotechnology medicines and advanced therapeutic medicinal products (ATMPs) [5]. With this in mind, the improvement of our understanding of epithelial tissue in healthy and diseased states can reveal novel strategies to maximise effective drug delivery and identify targets for the management or treatment of chronic disease. In order for this objective to be realised, complex, physiologically-representative *in vitro* models must be developed to address the inadequacies of current model platforms.

The use of biomimetic tissue-engineered scaffolds as a three-dimensional (3D) model is one approach by which such sophisticated models might be achieved. If designed appropriately, these constructs can incorporate an extracellular matrix analogue, pertinent cell populations and the appropriate signalling factors in the correct architectural arrangement to produce an *in vitro* system that achieves complexity and microscale structure that accurately reflects multicellular organs [6]. This is in contrast to current *in vitro* models of epithelial barriers, which typically consist of an epithelial monolayer cultured on polymeric cell inserts [7,8]. Although these models are useful for studying transepithelial transport of molecules and barrier function, they are ultimately an oversimplified model that can contribute to incomplete assessment of novel drug candidates and drug delivery technologies prior to animal testing; this increases the likeliness of failure at later stages in drug development [9]. Additionally, culture on polymeric inserts can alter cell growth and phenotype, with different proliferation, protein expression and cellular differentiation observed when different compositions of natural and synthetic substrates are utilised [10–13]. Early work by Davenport and colleagues, for example, highlighted that the presence of a type I collagen substratum induced ciliation within primary rat tracheal epithelial cells cultured at an air-liquid interface (ALI) that could not be achieved by culture on polycarbonate inserts [10]. The coating of collagen I hydrogels with type IV collagen and laminin, two basement membrane components, was found to alter basal cell attachment of oral epithelial cells and differentiation into a more mature mucosal barrier [11]. Similarly, laminin coating of poly-DL-lactic acid films enhanced expression of alveolar epithelial markers in attached embryonic stem cells [12] and influenced the barrier integrity and pigmentation in stem cell-derived retinal epithelial cells cultured on coated tissue culture plastic [13]. These examples demonstrate the applicability of natural polymers on epithelial functionality and highlight the potential of using extracellular matrix (ECM)-inspired substrates for *in vitro* culture. Furthermore, they lend credence to our hypothesis that the use of natural biomaterials homologous to the *in vivo* ECM can provide the most organotypic cell culture model and, combined with appropriate 3D tissue architecture, provide an advanced epithelial culture system.

Respiratory tissue of the tracheobronchial region in the airways is composed of a pseudostratified epithelial layer containing three main cell types-ciliated epithelial cells, goblet cells and basal cells-that are supported by a multi-cellular, fibrocartilaginous tissue basolateral to the epithelial cells [14,15]. For tracheal tissue engineering, three critical factors have been identified for successful recapitulation of the large airways: a basal lamina equivalent containing collagen fibres, mesenchymal cells such as fibroblasts, and the presence of an ALI system [16]. Indeed, a tissue-engineered approach towards *in vitro* modelling with type I collagen hydrogels has been shown to support the growth and differentiation of respiratory epithelial cells in co-culture with fibroblasts in a number of studies (reviewed in Ref. [9]). Recent interest has also arisen in the provision of naturally-derived ECM scaffolds for 3D airway modelling through the application of decellularised (DC) tissue either as DC tracheae [17], tissue slices of whole DC lung [18–21] or even the entire DC lung itself [22]. However, beyond the research of Omori and colleagues [23–25], there are few studies that use porous polymeric collagen sponges to provide a better *in vitro* representation of the fibrous tracheobronchial tissue architecture. Of course, it is desired in such porous materials that the epithelial cells do not migrate into the core of the construct and maintain their presence instead as a barrier at the scaffold surface as an interface. Thus, in this study we report on the fabrication of a bilayered collagen-based scaffold that permits the culture of bronchial epithelium on a two-dimensional (2D) film layer and the co-culture of fibroblasts in 3D in a porous layer beneath the epithelia.

The bilayered collagen scaffold proposed in this study is composed of a lyophilised collagen-hyaluronate co-polymer suspension, combining a film top-layer and porous sub-layer. Collagen membranes and hyaluronan-derivative films have shown promise for respiratory epithelial culture [16,26] but to date they have not been investigated as a co-polymer film to combine the benefits of each macromolecule alone. Regarding the sub-layer, porous collagen-hyaluronate scaffolds, previously designed by our group for cartilage regeneration, have been shown to facilitate cell growth and chondrogenic differentiation with mesenchymal stem cells [27–29]. The pore size of these scaffolds can be tailored by altering the freezing temperature of a controlled lyophilisation process which itself can influence cell adhesion, migration and growth in the construct [30–32]. These scaffolds were incorporated as the sub-layer of our epithelial *in vitro* substrate for co-culture of fibroblasts. Finally, type I collagen and hyaluronate are the predominant constituents of the tracheobronchial respiratory tract [15], and therefore the bilayered collagen-hyaluronate scaffold can further recapitulate the ECM of the tissue that is being modelled.

Thus, the major objective of this study was to engineer a bilayered collagen-hyaluronate scaffold as a tissue-engineered template for the development of a physiologically-representative 3D *in vitro* tracheobronchial epithelial co-culture model. To achieve this, this scaffold was firstly fabricated, characterised and subsequently assessed for its feasibility to support the growth and differentiation of a bronchial epithelial cell line. Finally, the scaffold was examined for its ability to support the co-culture of the epithelium with a fibroblast cell line in order to validate the scaffold as a template for 3D respiratory epithelial *in vitro* culture systems.

## 2. Materials and methods

### 2.1. Scaffold fabrication

#### 2.1.1. CHyA film fabrication

CHyA films were fabricated using a modification of a previously described method [33]. In brief, a suspension of collagen and hyaluronate was dehydrated under airflow. The suspension of 0.5% microfibrillar bovine tendon collagen (Integra Life Sciences, Plainsboro, NJ) and 0.044% hyaluronate sodium salt derived from *Streptococcus equi* (Sigma–Aldrich, Arklow, Ireland) in 0.5 M acetic acid (Sigma) was first blended at 15,000 rpm and 4 °C for 3.5 h using an Ultra Turrax T18 Overhead blender (IKA Works Inc., Wilmington, NC) and subsequently degassed under a vacuum to remove all air bubbles created from the homogenisation process. The slurry suspension was pipetted onto a 12.5 × 12.5 $cm^2$

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