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Double thin film-based sandwich-cell carrier design for multicellular tissue engineering

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ABSTRACT

Many organs are multicellular and each cell type requires a different microenvironment. Thus, there is a need for modular structures where the microenvironment of each cell type can be tuned separately. Herein, we describe enzymatically crosslinked gelatin based double layered film structures where each layer can be loaded with growth factors separately. As a model, we have developed a bi-layer system to produce a respiratory epithelium. This system constitutes an *in vitro* "epithelial patch" that can be adhered to the lumen of any implant. Crosslinking of the patches with transglutaminase resulted in 7 days of stability at 37 °C. The film layer was first used to release growth factors and it was shown that the release significantly improved the proliferation over 5 days. A549 human lung epithelial cells were used and under the release of an epithelial growth supplement mix, there was a significant improvement on the epithelial proliferation ($p < 0.01$). The designed substrate was successfully attached to titanium implants and we demonstrated the stability of the epithelial patch under *in vitro* conditions for 7 days without deterioration. Under co-culture conditions for three days both cell types were alive. Such patches can be used to obtain fast epithelialization of large implant surfaces.

1. Introduction

During, the embryonic development in vertebrates, the invagination of blastophore in the gastrula leads to three layers: the endoderm is the inner layer, the ectoderm is the outer layer and the mesoderm is the middle layer. In organogenesis all organs developed from these 3 layers of cells. The nervous system, the skin and hair originate from the ectoderm. Bone, muscles and vascular system are developed from the mesoderm. The endoderm leads to the formation of the lining of tube shaped organs like part of digestive tract or respiratory tract. The association of mesodermal cells and endodermal cells and their differentiation provides a functionality to different organs such as respiratory tract or urinary bladder.

A clinically relevant example is the tracheal respiratory epithelium which is a pseudostratified ciliated columnar epithelium that contains five different cell types: 1) ciliated columnar cells, 2) goblet cells, 3) basal cells, 4) brush cells, and 5) dense core granulation cells [1]. The ciliated columnar cells are elongated cells with apical cilia responsible for moving mucus along the surface of the epithelium. Goblet cells are disseminated individually within the epithelium, they are mucus secreting exocrine cells that produce the large part of the mucus covering the epithelial surface [2]. The basal cells are rounded cells located in the basal surface of the epithelium. Moreover, they are the reserve stem cell population which replaces lost ciliated cells and goblet cells. The brush cells are columnar cells with apical microvilli instead of cilia, they synapse with the dendritic endings of sensory nerve fibers playing a sensory receptor role. The dense core granule cells are endocrine cells that release vasoactive substances [3]. All these cells types are lined on the lamina propria which consists in a thin layer of fibroelastic connective tissue rich in elastic fibers. The collagen and glycoproteins in the basement membrane are produced in a part by the epithelial cells and in other part by connective tissue especially by the fibroblasts [4].

The respiratory epithelium has several dynamic functions such as 1) warming the inspired air by heat conduction from blood flow in the vascularized connective tissue; 2) moistening inhaled air by evaporation of water from mucous or serous glandular secretions; and 3) removal of the exogenous particles such as dusts or bacterial materials by trapping them in the sticky mucous layer and then transport them

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to hypopharynx by the ciliary movements [5]. This overview of the respiratory epithelium's functions demonstrates that the deficiency of one of these roles may lead to pathological situations. For example, the primary ciliary dyskinesia that affects the cilia motility or the cystic fibrosis leads to a thick and viscous mucous secretion responsible for respiratory infections because of mucus stagnation [6].

The complexity of these physiological functions makes the development of tracheal substitutes more difficult. Indeed, although the need to develop a tracheal substitute, to cure expansive tracheal defects, is not new [7], the optimal technique or biomaterial that can be used in routine clinical applications have not been found yet. This is due to the structure and composition of the trachea itself which is a multi-cellular organ composed of cartilaginous, connective and epithelial tissues [8]. Mimicking the interactions of the connective tissue ECM secretions and with the epithelial lining is an important point to consider in tissue regeneration [9]. All the different techniques using prosthetic replacements, allografts, composite autografts, proposed over the years to replace trachea have shown limitations [10]. The absence of functional respiratory epithelium in these current solutions has been considered as one of the limits in prosthetic replacement and tissue grafts. The main problems following the lack of epithelial layer are bacterial and/or fungal colonization of the prosthesis lumen, mucous plugs, and connective tissue hypertrophy resulting in endoluminal stenosis [11]. A typical example clinical issue concerned by these problems in head and neck surgery is the replacement of larynx.

The late stage laryngeal carcinomas are most commonly treated by laryngectomy. Separation of digestive and respiratory tracts results from this surgical intervention with a definitive tracheostomy and the loss of the capacity of phonation. These changes deteriorate the patient's life quality significantly. One of the working axes to remedy this situation is the development of an artificial larynx, where titanium can be utilized due to its mechanical stability [12]. Our group has recently reported the first successful implantation of such a system clinically [13]. This artificial larynx is designed in two parts: a multifunctional valve to replace the larynx's function itself (particularly the function of epiglottis) and a tracheal substitute to connect remaining trachea and the multifunctional valve. For this tracheal substitute we developed a porous microbead-based titanium implant that provides better integration with host tissues [14]. The integration happens through the migration of the cells from the surrounding connective tissue into the porous titanium structure. But in animal studies, we were confronted with the same problems such as endoluminal stenosis and mucous plugs, both phenomenon due essentially to the absence of re-epithelialization of the endoluminal side of the titanium implants [15]. In *in vivo* animal trials we could observe often spontaneous epithelialization only in the case of small animals like rats [16]. But there was no epithelialization either in the case of bigger animals such rabbits and sheep, nor in human transplantations [13], even though no side effects due to this absence was observed. Thus, the further clinical improvement of the artificial larynx seems to depend, in part, to the ability to regenerate a functional respiratory epithelium.

In order to solve this problem we propose a multilayered epithelial cell delivery system, where separate substrates for the epithelial cells and underlying connective tissue can be delivered simultaneously and where each component of the carrier can be modularly produced [17]. For this end, we developed a double layered crosslinked gelatin film based constructs where the physical and chemical properties of the parts facing the epithelial cells or the connective tissue cells can be separately controlled (Fig. 1).

Gelatin, a natural polymer which is obtained by denaturation of collagen, is a widely used biocompatible material. Its ability to form thermoreversible gels and the presence of RGD sequences within its chains due to its collagenous origin, makes it a versatile material for development of tissue engineering scaffolds, delivery systems and cell growth substrates [18,19]. However, gelatin is highly soluble in aqueous solutions and gelatin based films need to be stabilized for long-term applications. There are several ways to crosslink gelatin such as use of chemical crosslinking agents like EDC/NHS or genipin. A more versatile and specific way is the use of transglutaminase enzymes, which in nature crosslink collagen molecules [20]. To reach this purpose, we emit the hypothesis that a functionalized biodegradable gelatin hydrogel patch (where the degradation is controlled by crosslinking degree *via* transglutaminase) could support the epithelial cell grafts until degradation of the synthesized material by connective tissue. These hydrogels will serve as a feeder layer and will release growth factors by mimicking an artificial basement membrane. This basement membrane can be further supported by an additional patch layer containing connective tissue cells to mimic the actual epithelial layer. In order to test the efficacy of such a design we tested the stability, controlled release capacity and feasibility of the system as a multicellular delivery system with model cell types.

2. Materials and methods

2.1. Materials

Gelatin from porcine skin and BSA^{FITC} ($Mw = 6.6 \times 10^4$ Da, pI = 4.7–4.9) were purchased from Sigma-Aldrich (Germany). For cell culture Phosphate Buffered Saline (PBS), RPMI 1640 cell culture basal medium, penicillin/streptomycin mixture, fungizone, and trypsin–EDTA were obtained from General Electrics Healthcare (USA). Fetal Bovine Serum (FBS) was provided by Gibco (France). SupplementMix airway epithelial cell growth medium was purchased from PromoCell

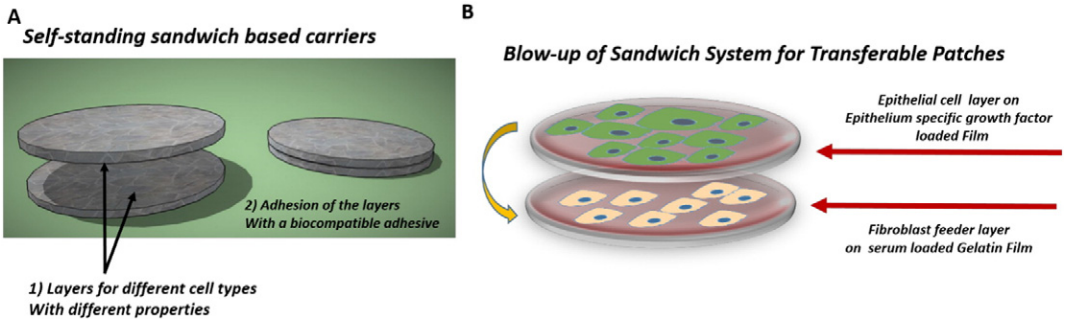

Fig. 1. Sandwich like double-layered patches for transfer of epithelial cells in the presence of a connective tissue. The two layers are put together with a wet, non-cytotoxic adhesive which provides stability over a week. Each layer has its own physical properties adjusted for the cells they carry: *i*) the upper layer will contain the epithelial cells and specific growth factors loaded and *ii*) the bottom part will integrate fibroblasts with serum components loaded.