35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

A Dynamic Perfusion Bioreactor Approach for Engineering Respiratory Tissues In-Vitro*

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***Abstract*— *In vitro* culture of respiratory tissues poses many challenges due to the intrinsic complexity of the respiratory system. Multiple cellular phenotypes comprise the respiratory epithelium and operate under dynamic, gas-interchanging conditions that should be replicated for near-physiologic cultivation of functional tissues *in vitro*. A novel biomimetic perfusion bioreactor system has been proposed to reconstitute key functional conditions of the human lung. This portable system consists of several biologically-inspired components: (i) a 3-dimensional (3-D) elastomeric soft tissue scaffold construct, (ii) a mechanical actuator, (iii) a perfusion system and (iv) gaseous exchange capabilities. These integrated components operate synergistically to create a unique, dynamic air-liquid interface (ALI) environment that allows controlled application of physiological and pathological strain while complementing standard cell culture techniques. This system holds potential for engineering 3-D tissues to meet growing demand for a range of applications, from more ethical and efficient pharmaceutical screening to clinical graft transplants.**

I. Introduction

Destructive respiratory diseases are an increasingly significant global cause of morbidity and mortality collateral to increasing trends in smoking and airborne pollutants [1]. Acute and chronic respiratory diseases range from simple and reversible irritation (e.g. asthma), to debilitating and fatal conditions (e.g. emphysema). Despite the high global prevalence and economic burden of respiratory disease, there are no current cures as lung tissues do not repair and regenerate beyond a cellular level [2]. Thus from the point of disease onset, lung tissues are progressively destroyed and the sufferer's ability to breathe is increasingly restricted over time. Current management of chronic respiratory diseases includes drug therapy, oxygen therapy, surgery and pulmonary rehabilitation. End stage sufferers require ventilation assistance and ultimately, lung transplantation [3]. However, whole or partial lung transplantation remains a costly last-resort option associated with low success rates due to immune rejection and generally poor organ viability, as well as being further offset by severe donor shortages [4, 5].

Tissue engineering approaches have the potential to not only regenerate human respiratory tissue equivalents for clinical implantation, but also to provide an ethical research platform for disease pathology research, toxicology studies and more efficient pharmaceutical screening [6, 7]. The underlying principle of tissue engineering is the combination of living cells, a tissue scaffold and biologics to stimulate cell growth and proliferation in a simulated biological environment. Although many cultured respiratory cell lines exist, successful engineering of respiratory (particularly lung) tissues *in vitro* has been limited. Progress has been hindered by challenges in maintaining respiratory cell phenotypes *in vitro*, and in developing scaffolds that adequately capture the complex 3-D architecture and biomechanical properties of lung tissues *in vivo* [8].

Increasing understanding of the physiological conditions and properties of the tissue/s of interest aids advancement. For example, the critical roles of mass nutrient transfer and mechanical stimulus in cell differentiation and function are now increasingly recognized [9-11]. Such knowledge shifted the previous paradigm of static cell culture and prompted the development of bioreactor technologies to provide improved and more sophisticated simulation of *in vivo* environments *in vitro* [12, 13]. This research promotes the most recent tissue engineering paradigm of utilizing a biomimetic perfusion bioreactor approach in the development of a multifactorial system capable of culturing lung tissues *in vitro*.

II. Biomimicking the Human Respiratory System

In order to formulate a design process for engineering tissue, it is necessary to first understand the anatomy, physiology and cellular environment of the tissue of interest in both normal and diseased states. The human respiratory system is responsible for meeting the oxygen requirements of the body by the continual exchange of oxygen and carbon dioxide between the lungs, circulatory system and environment. The respiratory tract is composed of an upper and lower portion, with nearly 50 distinct cell types and their respective physiological functions. From the trachea, the respiratory tract repeatedly bifurcates into increasingly smaller airways that end with clusters of interconnected sacs known as alveoli. The upper respiratory tract primarily conducts and conditions air, whilst gaseous exchange occurs in the lower respiratory tract at the alveoli.

The structure of an individual alveolus is relatively simple. The alveolar wall consists of an epithelium overlying a thin stroma which contains fibroblasts, myofibroblasts, extracellular matrix components and endothelial capillary cells. The alveolar epithelium itself consists of two cell types: type I and type II pneumocytes. Alveolar type I cells are squamous and comprise about 90% of the epithelial surface area, the remaining 10% being made up by the smaller cuboidal type II cells. Type I pneumocytes fuse with the capillary endothelium to formulate the blood-air exchange barrier [14], whilst type II cells secrete surfactant to prevent alveolar collapse [15].

Theoretically, any section of the respiratory tract can be engineered, from a relatively simple portion e.g. the bronchial

This research is supported by Biometic Pty Ltd.
Christine Poon is a recipient of the Australian Postgraduate Award (APA).
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lumen wall to more holistic functional tissue containing alveoli and branching airways. This study focuses on the distal alveolar portion of the lung, which experiences very intricate biomechanics during breathing.

Breathing is a highly dynamic process that occurs as a result of an elaborate biomechanical sequence controlled by the autonomic nervous system. During normal inspiration, contraction of the diaphragm and intercostal muscles of the ribcage causes expansion of the thoracic cavity. This creates a negative pressure differential between the intrapleural cavity and atmosphere, which in turn causes the alveoli to expand and thus air is drawn into the lungs in accordance to Boyle's law:

$$P_1V_1 = P_2V_2$$

where P = pressure and V is the volume of a container. The converse occurs during expiration, which commences when the diaphragm and respiratory muscles relax; this reduces the thoracic volume and thus air is expelled from the lungs.

The alveolar epithelium can be understood to undergo periodic wetting during breathing– dry during inhalation when strained and exposed to air, and remoistened by extracellular fluids during exhalation and relaxation of the alveoli [16]. The alveoli are often described as 'grape-like' individual sacs when in fact, the tissue more closely resembles a sponge with interconnected pores. Within each acinus (cluster of alveoli), the alveoli are connected via a shared septal wall and have an average pore diameter of 200-300µm [17]. During breathing, alveoli are not equally inflated but are instead recruited along a pressure gradient [18]. It follows that there is a physiological range of strain values experienced by lung tissues, which is reported to be between approximately 5-15% for healthy lungs [19, 20]. Application of increased strain to *in vitro* cultures has been shown to damage tissue, mimicking pathological conditions in destructive lung disease, with values between 15-25% [19, 21]. It is therefore important to consider the magnitude of strain applied to mechanically actuated cell cultures as there may not be a significant difference between physiological and pathological values.

During inhalation, the airways are enlarged as the luminal wall is stretched outwards radially along with the expansion of the lungs. Larger diameter airways have decreased resistance to air flow, hence inhalation is relatively shorter than exhalation. Conversely, during exhalation, airway resistance increases as the diameter of the airways decrease. This phenomenon contributes to the inspiratory to expiratory ratio (I:E ratio)- a ratio of the time spent in inhalation and exhalation during one breath. In normal individuals, the I:E ratio is approximately 1:1.5 – 1:2, whereas in people with lung diseases that cause increased airway resistance e.g. asthma, the I:E ratio can be between 1:3 – 1:8 [22, 23]. Thus tissue-specific waveform patterns of strain should be considered in addition to magnitude to optimize capturing physiological conditions *in vitro*.

Respiratory epithelial cells are routinely exposed to varying gas compositions during breathing (Table 1). This presents a greater challenge to simulate than for tissues that experience lower oxygen exposure *in vivo* solely via the circulation, e.g. muscle or bone. As mammalian cultures are usually submerged, gas composition and humidity are rarely considered and incubators typically maintain a constant air composition of 5% CO_2 and humidity of approximately 85-95% at 37ºC [24]. However, pulmonary surfactant quality and pneumocyte function have been shown to be significantly compromised by submerged culture (at 100% humidity) compared to 20% humidity culture [25]. In addition, hypoxic and hyperoxic environments are known to cause oxidative stress and cellular damage [26]. Tailoring gas composition and humidity may therefore be particularly critical given the unique gas environment of the respiratory epithelium.

TABLE I. TYPICAL COMPOSITION OF INSPIRED (ATMOSPHERIC) AND EXPIRED AIR [27]

Component	*Atmospheric air (%)*	*Expired air (%)*
N_2 (and inert gases)	78.62	74.9
O_2	20.85	15.3
CO_2	0.03	3.6
H_2O	0.5	6.2
Total	100	100

In the lung, alveolar epithelial cells receive nutrients via the dense capillary network lining the basement membrane, and extracellular fluids (ECF). Nutrients and metabolites are exchanged between the epithelium, ECF and capillary blood by passive diffusion and active transport mechanisms. Perfusion rate within the lung varies depending on tissue health, gravity, geometrical location (e.g. apex of the lung), regional vascularity and even within one breathing cycle due to phase-dependent tissue strain. Values described for the lung are approximately 300-400mL/100g tissue/minute [28].

Orientation of nutrient source with respect to the cells is another consideration. Basolateral exposure of cells to nutrient-rich media in ALI culture more accurately captures the alveolar epithelium conditions compared to exposing cells to nutrients from all directions as in submerged culture [29, 30]. Many chemically-defined media formulations e.g. small airway growth medium (SAGM) are commercially available for respiratory cell studies.

Table 2 below summarizes physiological properties and conditions that formulate key parameters to be replicated in the design and development of a biomimetic system that would provide optimal, near-physiologic conditions for culturing lung tissues *in vitro*.

TABLE II. DESIGN INPUTS BASED ON BIOLOGICALLY-INSPIRED PROPERTIES OF LUNG TISSUE (*VALUES BASED ON HEALTHY TISSUE)

*Parameter**	*Physiological Data Values*	*Proposed System Values*	*References*
Tissue strain	5-15%	5-15%	[19, 20]
Breathing rate (at rest)	12-15 breaths per minute	12 cycles/minute (0.2 Hz)	[27]
I:E ratio	1:1.5-1.2	1.6:3.3 (seconds)	[22]
Alveolar diameter	200-300 µm	Scaffold pore size of 200-300 µm	[17]
Temperature	37±0.5 ºC	37ºC	[24]
O_2 (inspired-expired)	20%-15%	20%-15%	[27]
CO_2 (inspired-expired)	0.03%-3.6%	0.05%-5%	
Perfusion rate	Variable	0.01-0.1mL/min	-

III. Design Concepts

The current design can be considered as an integrated assembly consisting of: (i) 3-D biocompatible soft tissue scaffold construct, (ii) mechanical actuator (Biometic Pty Ltd's IAXSYS^{TM}), (iii) perfusion system and (iv) gaseous exchange unit. Please see Fig. 1 for a schematic overview of the system.

3-D scaffold construct for air-liquid interface culture

The biaxx^{TM} unit is designed to fit in a standard six-well culture plate and is composed of a 11x11x3mm elastomeric porous 3-D synthetic scaffold embedded in a flexible actuation arm configuration (Fig.s 1a, 2a, 2b). The scaffold has a porosity of >95% and pore size range of 20-300µm (Fig. 2b). To mimic exhalation, the scaffold rests in a cup conformation submerged and moistened by culture media. Linear strain applied to the unit is translated into multi-axial stretch of the scaffold whilst simultaneously raising it above the media into air, mimicking inhalation conditions. This cyclical motion is achieved via magnetic coupling between the dynamic endplate of the unit with the IAXSYS^{TM} mechanical actuator (Fig. 1e). Apart from the magnet, the entire unit including the scaffold is fabricated from a proprietary biocompatible and biodegradable polymer blend. A preliminary study by Poon et al confirmed cell attachment, growth and proliferation of H460 human lung carcinoma cells on this dynamic unit [31].

Mechanical Actuation

A servo motor drives a magnetic bed containing six magnets spatially arranged to actuate six corresponding biaxx^{TM} units situated within a standard six-well culture plate that sits on the IAXSYS^{TM} (Fig. 1c). Custom waveforms, amplitude (linear displacement of the magnet bed) and frequency can be applied via the system's software (Fig. 1f), e.g. a sawtooth waveform of 1:3 with a frequency of 0.2Hz may be used to mimic normal I:E ratio and breathing rates.

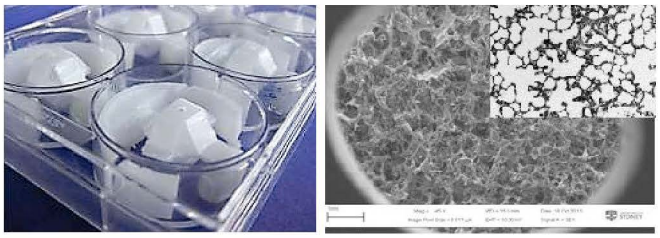

Figure 2. (a) Completed biaxx^{TM} units; (b) SEM image of scaffold microstructure (45x magnification). Inset: histological section of distal lung tissue to highlight the structural similarity between the scaffold and alveoli.

Perfusion

Perfusion of a tissue-engineered construct mimics capillary blood flow through an organ in nutrient delivery and waste removal. Perfusion systems are increasingly used for developing 3-D constructs as passive diffusion in static culture is insufficient for effective nutrient and waste transfer to the center of the scaffold, resulting in a necrotic core [32, 33]. Perfusion requirements are tissue-specific. If flow-mediated shear stresses are too high, cells may be damaged and/or flushed off the scaffold. At present, the optimal rate for lung tissues is yet to be determined. Many variables including scaffold porosity, geometry, material, degradation kinetics, media viscosity and cell characteristics (e.g. growth rate) can affect the magnitude and distribution of fluid shear stress within a construct. Therefore, results may vary between studies depending on the model used. Initial perfusion input ranges suitable for the scaffold architecture in this study are currently being investigated by computational modeling of the biaxx^{TM} scaffold and will be later refined experimentally. The proposed perfusion system works to continually circulate media between a standard six-well culture plate and external media reservoir via a pulsatile pump (Fig. 1b). The tubing configuration clips onto the culture plate lid, which is modified to allow syringe access into the well. Current efforts involve optimizing tubing configuration and length while maintaining equalized flow rate to the culture plate wells.

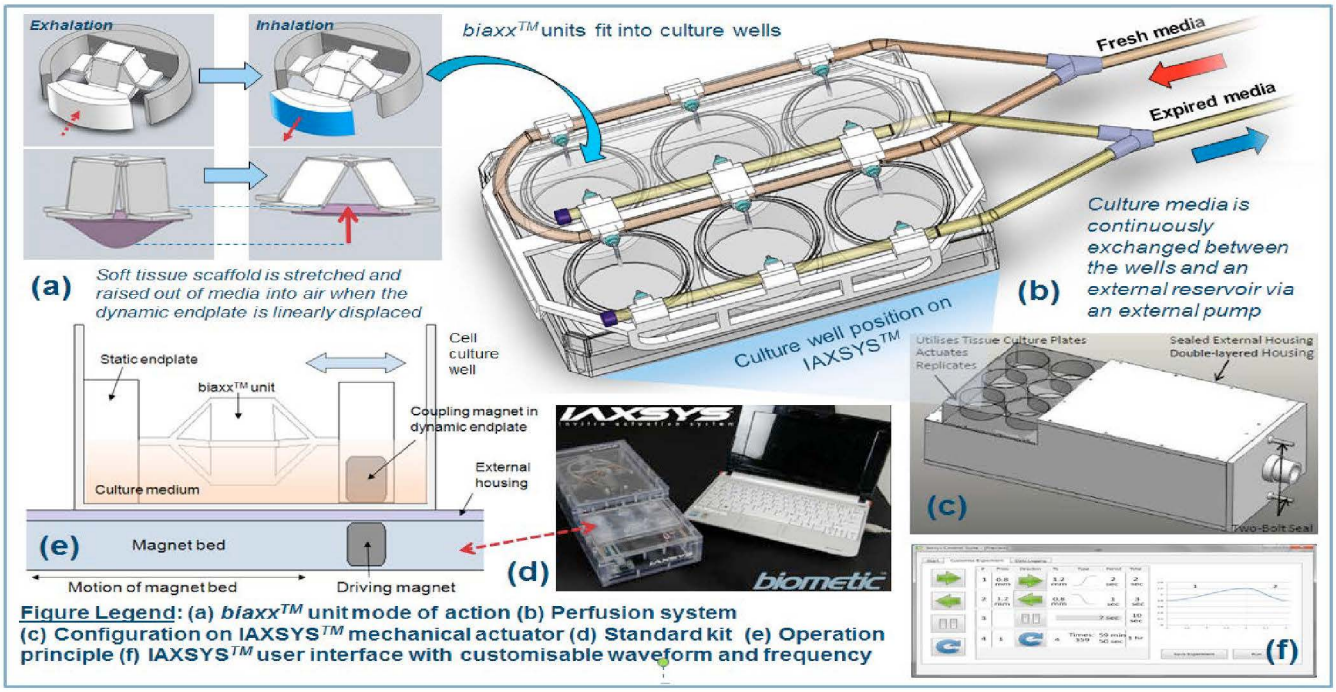

Figure 1. Proposed system provides ALI culture conditions, mechanical stimulus, perfusion and gas exchange for 3-D culture of lung tissue

Gas exchange

Although it is possible to modify the CO_2 injector and humidity regulator settings in a standard laboratory incubator to provide appropriate air-culture conditions, the aim of this study is to develop a system that is compatible with standard laboratory equipment, including default settings. The simplest concept is to house the entire system in Figure 1 within a sterile air chamber. An alternative air-delivery mechanism paired collaterally with the media perfusion system employing gas-permeable tubing to emulate appropriate gas-exchange and air-flow dynamics has also been proposed.

IV. Conclusion

Due to the complex biomechanics of the respiratory system, it is evident that a multifaceted approach is required to address the current limitations of static, submerged culture techniques in engineering functional respiratory tissues. In this study, an integrated system capable of providing near-physiologic respiratory conditions has been proposed. Although this novel bioreactor configuration was in prototyping stages at the time of publication and requires further development and validation, such a system has the potential to provide an ideal, biomimetic environment for reconstituting functional lung tissues *in vitro*.

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