# LUNG SCAFFOLDS FOR BIOENGINEERED ORGAN.

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**Abstract:** The main diseases of the respiratory system such as chronic obstructive pulmonary disease, emphysema, idiopathic pulmonary fibrosis and primary pulmonary hypertension, results in an irreversible structural damage in the lung parenchyma, and lung transplantation the only indication. Unfortunately, the success of lung transplantation is limited, due to limited number of organ donors and the incidence of bronchiolitis obliterans resulting in an alloimmune response elicited by disparities between donor and recipient antigens. In this context, bioengineering lungs is considered a potential therapeutic approach. The decellularized organs matrix potentially maintains the three-dimensional architecture and biochemical composition, as well as the original tissue microvasculature. This ability makes the decellularized lung promising for generation of functional bioartificial lungs. This study aims to demonstrate in an experimental animal model the decellularization process of the lungs in order to prepare scaffolds for bioengineered artificial organ.
**Keywords:** Scaffolds, Decellularization, Recellularization, Lung, Bioengineering organs, Biomedical Engineering.

## Introduction

Significant respiratory diseases such as chronic pulmonary obstruction, emphysema, idiopathic pulmonary fibrosis, primary pulmonary arterial hypertension, interstitial lung disease, cystic fibrosis and α-1-antitrypsin deficiency result in irreversible structural lung damage, with lung transplantation as the only therapeutic indication when the disease reaches an advanced progression [1].

The success of lung transplantation is limited, mainly due to the paucity of lung organ donors and the incidence of obliterative bronchiolitis, which result from an alloimmune response caused by disparities between the donor's and recipient's human antigens. In effect, the 50% survival rate of patients after lung transplantation is currently confined to around 5 years [1].

The current limitations of donor's lung require, therefore strategies to increase the availability of suitable organs for transplantation.

This need is enhanced by the progressive ageing of the population which increases the waiting lists of patients with severe respiratory diseases. In this context, the bioengineering of lung is considered as a potential therapeutic alternative, but current research are in preliminary stages and intensive scientific efforts are therefore required [2].

The decellularization technique has been previously used for engineering a diversity of tissues, including the skin, esophagus, artery, bladder, trachea and heart. Compared to other organs, the structure of the lung is particularly complex, and this increases the difficulty of the bioengineering process. Although this complexity, it has been demonstrated that with the use of suitable protocols, the lung may be completely decellularized to obtain an intact acellular structure.

## Materials and methods

**Lung decellularization –** In this experimental model, the lungs are obtained from male rats of the lineage C57BL/6, healthy (18-20g) 7-8 weeks old. The rats are anesthetized with urethane (1 mg/kg, intraperitoneal) and sacrificed by exsanguination following a protocol previously approved by the Ethical Committee for Animal Research of our institution [3].

Immediately after euthanasia, the diaphragm is removed and the rib cage opened exposing the lungs. Subsequently, the lungs and trachea are dissected and cleaned to remove any attached esophageal, lymphatic, and connective tissues. Then, lungs were maintained in a 50 ml polystyrene conical tube with 5 mL of PBS, frozen and kept in a -80º freezer until the beginning of the protocol.

The decellularization process (Figure 1), adapted from Cortiella et al. (2010) consist of 6 steps, harvesting, cleaning, freezing and thawing, sodium dodecyl sulfate (SDS) washing and PBS rinsing [4].

Firstly, the lungs were subjected to 4 freezing/thawing cycles of 10 minutes each in a -80°C freezer and a 37°C warm bath.

Secondly, the lungs were washed 6–8 times with an 2 mL syringe of PBS instilled, slowly and allowing the liquid to return, by the trachea - so as not to cause damage in lung structure - followed by 2.5 mL of deionized water.

In the third step, the lungs are instilled via tracheal with 2.5 mL of 1% sodium dodecyl sulfate (SDS) detergent and are subsequently agitated for 24h at room temperature in a 50-mL polystyrene conical tube with 3 mL of 1% SDS. In step 4, the lungs were maintained overnight in 1% SDS at room temperature without agitation. To finish the process of obtaining acellular lung scaffolds (Figure 2), the lungs were rinsed with 2 mL of PBS and maintained in 3mL PBS in agitation for 24h in step 5, and were maintained in PBS without agitation in step 6.

To verify effective cell removal, the decellularized lungs will be sectioned and stained with DAPI (4'-6-diamidino-2-phenylindole), Propidium Iodide or Hoechst, and examined under the microscope.

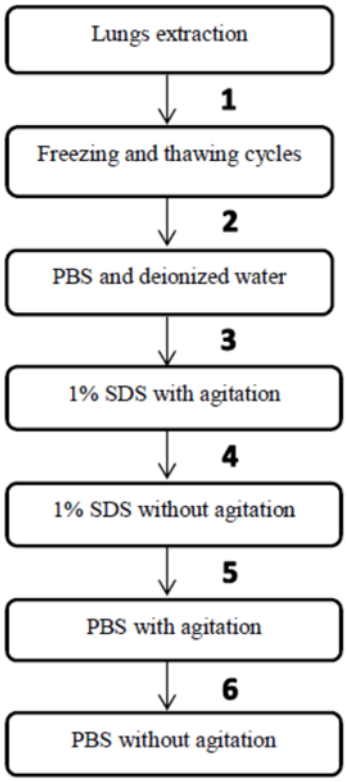


Figure 1: Steps of decellularization protocol.

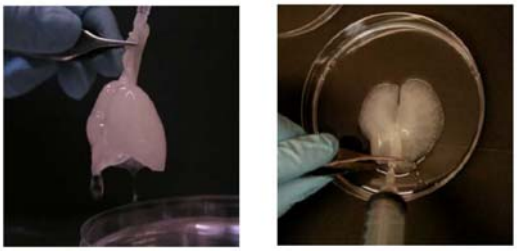

Figure 2: Images of a decellularized rat lung.

**Preparation of decellularized lung slices –** A mixture of Optimal Cutting Temperature compound (OCT, Sakura) and PBS at a 3:1 ratio is infused via the trachea into decellularized lung matrices, which then are embedded in OCT and snap-frozen in liquid nitrogen. Frozen samples are stored at -80ºC and subsequently, cryosections (40-100 µm) of frozen lung samples will be obtained in a cryomicrotome and placed on top of a cell culture plate or a stretchable membrane. The slices are thoroughly washed with PBS containing antibiotics to remove the OCT.

**Quantification of deoxyribonucleic acid (DNA) in decellularized lungs –** DAPI staining is used to verify the absence of nuclei or DNA after the process of decellularization. For staining with DAPI, a DAPI stock solution (5 mg/mL; 14.3mM for the dihydrochloride or 10.9mM for the dilactate) will be made by dissolving 10mg of DAPI in 2mL of deionized water, following sonication for 2 h. The images of these samples ensure that the various areas of the sample are free from cells after decellularization. Moreover, the amount of DNA remaining in the decellularized samples was extracted as previously described [4]. Samples are separated by electrophoresis on a 3% LMP agarose gel with ethidium bromide at 60 V for 1 h, and observed with ultraviolet transillumination.

**Quantification of extracellular matrix (ECM) components in decellularized lungs –** Collagen, elastin, laminin and glucosaminglycans (GAGs) are quantified in decellularized lungs. For the sake of comparison, this study will be also carried out in fresh (non-decellularized) lungs. Collagen is quantified by oxidation of 4-OH-L-proline to pyrrole and reaction with p-dimethylaminobenzaldehyde (absorbance read at 560 nm). Elastin is quantitatively measured using the Fastin Elastin quantitative dye-binding kit (BiocolorLTD), according to the manufacturer's directions. Laminin will be measured using a laminin ELISA kit (Insight Genomics) on samples homogenized in protease inhibitor (Roche). The GAGs are determined using the dimethylmethylene blue assay. Total protein will be determined by the Bradford assay (SIGMA-ALDRICH).

**Scanning electron microscopy (SEM) –** Decellularized lung samples are prepared for their analysis under SEM by following a standard protocol for the preparation of tissue samples. Briefly, tissue samples are fixed with 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1M cacodylate buffer for 2 hours at room temperature, then rinsed in cacodylate buffer, sliced, and dehydrated through an ethanol gradient. Samples are further dehydrated in hexamethyldisilizane for 10 minutes and dried overnight, then sputter coated with gold and analyzed using the SEM.

**Two-photon microscopy –** Samples are viewed using multiphoton microscopy to detect tissue autofluorescence and second harmonic generation microscopy to detect fibrillar collagen. Fluorescence emission in the spectral region of 500–650nm are collected for detection of broadband autofluorescence from the lung. Second harmonic generation are collected using 800nm excitation and a 400 ± 14nm bandpass filter in the non-descanned detector path. Several sites at the apex of the lung lobe and the broncho-alveolar region are chosen. At each site, z-stack are obtained from the outer lung surface using a z-interval of 1 and 150 mm total depths, using a 40X, 0.75NA water immersion objective.

## Results and discussion

In our recent study demonstrated that the process of freezing and thawing, commonly used for the storage of decellularized organs did not induce significant changes in ventilatory mechanical properties of the lungs. Thus, the lung can be frozen after decellularization without changing its viscosity and elasticity for later recellularization [5].

The scientific literature shows that through the appropriate protocol, the lungs can be completely decellularized obtaining an acellular scaffold [6,7].

Some recent studies have reinforced the concept of a new breakthrough in the field of bioengineering lung. Price et al. (2010) [6] showed that decellularized lung can be recellularization with fetal lung cells submitted to simulated ventilation. In addition, Ott et al. [8] and Petersen et al. [9] have recellularized rodent lung scaffolds with alveolar and epithelial cells to obtain a bioengineered lung showing short-term functionality of gas exchange after in vivo implantation.

These studies reinforce the need for a protocol decellularization adequate in the literature, thus following in the advancement of bioengineered organ.

Although these works represent a milestone in the progress of lung bioengineering, any routine application in the future could be hampered by the difficulty of using differentiated cells, given their limited availability and proliferation capacity.

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