Human lung extracellular matrix hydrogels resemble the stiffness and viscoelasticity of native lung tissue

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Abstract

Chronic lung diseases such as idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD) are associated with changes in the extracellular matrix (ECM) composition and abundance affecting the mechanical properties of the lung. This study aimed to generate ECM hydrogels from control, severe COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD) IV) and fibrotic human lung tissue and evaluate if their stiffness and viscoelastic properties were reflective of native tissue. For hydrogel generation, control, COPD GOLD IV and fibrotic human lung tissue were decellularized, lyophilized, ground into powder, porcine pepsin solubilized, buffered with PBS, and gelled at 37°C. Rheological properties from tissues and hydrogels were assessed using a low load compression tester (LLCT) measuring the stiffness and viscoelastic properties in terms of a generalized Maxwell model representing phases of viscoelastic relaxation. The ECM hydrogels had a greater stress relaxation than tissues. ECM hydrogels required 3 Maxwell elements with slightly faster relaxation times ($\tau$) than that of native tissue which required 4 elements. The relative importance (RI) of the first Maxwell element contributed the most in ECM hydrogels whereas for tissue the contribution was spread over all 4 elements. IPF tissue had a longer lasting 4th element with a higher RI than the other tissues and IPF ECM hydrogels did require a 4th Maxwell element in contrary to all other ECM hydrogels. This study shows that hydrogels composed of native human lung ECM can be generated. Stiffness of ECM hydrogels resembled that of whole tissue while viscoelasticity differed.
Introduction

Chronic respiratory diseases are a prominent cause of morbidity and mortality worldwide (5) with chronic obstructive lung disease (COPD) being the 3rd leading cause of death in the United States. Chronic lung diseases, such as COPD and idiopathic pulmonary fibrosis (IPF), are characterized by extensive changes in the ECM, the 3-dimensional scaffold that provides mechanical and biochemical support/signals to cells. The ECM is often an under-recognized element in lung disease (4, 13, 29). However, increasing evidence suggests that the ECM plays an active role in lung pathophysiology.

Mechanical properties of the ECM dictate, in part, cellular responses to injury, with stiffness being explored most commonly (3, 14, 19). The lung ECM is a viscoelastic network of both elastic and non-elastic constructive fibrillar proteins embedded in a water-retaining gel of proteoglycans and glycosaminoglycans. Viscoelastic materials exhibit time-dependent strain often measured as relaxation when undergoing deformation (6). Viscoelasticity as a mechanical property influences cellular spreading, proliferation and differentiation, together with or independently of stiffness (7, 8). For the lung, which undergoes repeated stretch and relaxation, usually greater than 14,000 times each day, replicating the mechanical properties of the ECM is essential to accurately model the cellular environment in vitro.

Synthetic hydrogels have found their way into tissue engineering as ECM mimics. Natural hydrogels have been generated from e.g. decellularized human adipose (20), heart (15), liver (17) tissue and more (1, 9, 23, 25). As for the lung, hydrogels have been generated from porcine lung ECM (22). ECM hydrogels are generated from intact tissue by detergent based decellularization, gentle proteolytic solubilization (often with pepsin). Upon pH neutralization and bringing to physiological osmolarity, hydrogels form spontaneously at 37°C. As such, these hydrogels comprise the native ECM composition albeit not the macroscopic (micrometer-sized) architecture (10, 23).
In this study we report for the first time, the generation of ECM hydrogels from control and diseased (COPD GOLD IV and IPF) human lung tissues. The mechanical features of these human lung ECM hydrogels, as well as intact human lung stiffness and viscoelasticity were measured. Our data indicate that the ECM hydrogels partially replicate the mechanical properties of human lung tissue.

Materials and Methods

Processing of human lung tissue

Tissue from human explanted lungs were obtained through the Dept. of Pathology, remaining after diagnostic procedures, from control (non-usable donor lungs and tumor resection material) (n=13) and COPD GOLD IV (n=15) or IPF (n=12) patients undergoing lung transplantation or lung resection in the University Medical Center Groningen. The protocol was consistent with the Research Code of the UMCG, and national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies", http://www.federa.org and: https://www.umcg.nl/SiteCollectionDocuments/English/Researchcode/umcg-researchcode-2018-nl.pdf). De-identified control and IPF human lung tissue were provided by the University of Michigan; as the tissues were de-identified and coming from deceased donors, the University of Michigan Institutional Review Board deemed this work exempt from oversight.

Decellularization of human lung tissue

Lung tissues (Control n=3, COPD GOLD IV n=10 or IPF n=3) were minced using a blender, washed with demineralized H₂O (dH₂O), treated with trypsin (0.05% final conc., Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated (37 °C, 3h) (Figure 1a). The homogenate was repeatedly washed with dH₂O until the supernatant remained clear, before being sequentially treated with: saturated NaCL (6M) solution, 70% ethanol, 1% SDS solution, 1% Triton X-100, 1% sodium deoxycholate and DNAse 30µg/ml (in 1.3 mM MgSO₄ and 2 mM CaCl₂) solution, with 3 washes with dH₂O between treatments, each for 24h at
room temperature (RT) with constant shaking, except the enzymatic treatments which were
at 37 °C with shaking. The resultant decellularized ECM (dECM) was stored in sterile PBS
containing 1% penicillin/streptomycin at 4 °C. Decellularized tissues (3) were provided by the
University of Michigan (control n=6, IPF n=6).

Generating lung dECM hydrogels

The dECM samples were snap frozen in liquid nitrogen and lyophilized using a FreeZone
Plus lyophilizer (Labconco, City, Missouri, USA), then ground to a powder using an ULTRA-
TURRAX (IKA, Staufen, Germany). dECM samples from different donors from the same
disease group (Control n=9, COPD GOLD IV n=10 or IPF n=9) were pooled. The lung dECM
powder (20 mg/mL) was digested with 2 mg/ml porcine pepsin (Sigma-Aldrich, Saint Louis,
Missouri USA, Figure 1b) in 0.01M HCl with constant agitation at RT for 72 h. The digest was
centrifuged at 500g for 3min to remove any remaining undigested insoluble aggregates. The
pH was neutralized with 0.1M NaOH and brought to 1x PBS with one tenth volume 10x PBS:
this generated the pre-gel. Human lung ECM hydrogels were prepared in 48 well plates with
300 µl pre-gel per well at 37 °C for 1 h. Lung ECM gels were covered with 500 µl Hank's
balanced salt solution (Lonza, Verviers, Belgium) to prevent desiccation prior to mechanical
testing. Sections of lung ECM hydrogels were stained with hematoxylin & eosin (H&E) (12);
images were captured using a slide scanner (Nanozoomer 2.0 HT; Hamamatsu Photonics).

Protein distribution of whole, decellularized and pepsin digested
lung tissue

The protein content of native lung tissue, dECM powder and pepsin digested dECM (pre-gel)
was examined. 20 mg of whole tissue and dECM powder were solubilized in 1 ml RIPA
buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing 4 µl proteinase
inhibitor cocktail (Sigma-Aldrich, Saint Louis, Missouri USA) and 10 µl phosphatase inhibitor
cocktail (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 20mg of pre-gel was
prepared. The solubilized tissue, dECM powder and pepsin digested ECM solution were
mixed 1:1 with 2x sample buffer and separated on 5% and 10% SDS-PAGE gels. The gels were stained using Coomassie Brilliant Blue for 1 hour and destained with 50% methanol, 10% acetic acid. Images of the stained gels were subsequently digitized.

**Mechanical properties**

Fresh tissue (Control n=4, COPD GOLD IV n=5 or IPF n=3) and lung dECM hydrogels from control, COPD GOLD IV and IPF were subjected to stress relaxation testing using a low load compression tester (LLCT) at RT (Figure 1C), as described previously (24). The LabVIEW™ 7.1 program was used for the LLCT load cell and linear positioning for control and data acquisition. The resolution in position, load and time determination was 0.1 mm, 2 mg and 25 ms, respectively and the velocity of motion was controlled in feedback mode. The top plate moved downward (5 µm/s) until it experienced a counterforce of $10^{-4}$ N. Samples were deformed by 20% of their original thickness (Strain $\varepsilon=0.2$) at a deformation speed of 20 %/s (Strain rate $\dot{\varepsilon} = 0.2 \text{ s}^{-1}$). The diameter of the indentation probe was 2.5mm. The deformation was held constant for 200s and the required stress monitored. During compression, the required stress was plotted against the strain. In this plot a linear increase in stress as a function of strain was observed between a strain of 0.04 and 0.1, the slope of the line fit to this region was taken as stiffness (Young’s modulus). Since the stiffness of the viscoelastic gel depends on the strain rate, values reported here are valid only at a strain rate of 0.2 s$^{-1}$.

Stress relaxation, the required stress to maintain a constant strain of 0.2, continuously decreases with time, which is a clear indication of the viscoelastic nature of materials. The shape of the stress relaxation curve was mathematically modelled using a generalized Maxwell model (2)(Figure 1C). The continuously changing stress ($\sigma(t)$) was converted into continuously changing stiffness ($E(t)$) by dividing with the constant strain of 0.2. $E(t)$ was fitted to equation 1 to obtain the relaxation time constants ($\tau$), while equation 2 provided relative importance ($R_i$) for each Maxwell element.

\[ E(t) = E_1 e^{t/\tau_1} + E_2 e^{t/\tau_2} + E_3 e^{t/\tau_3} + E_4 e^{t/\tau_4} \]  \hspace{1cm} (1)
\[ R_i = \frac{E_i}{E_1 + E_2 + E_3 + E_4} \]  \hspace{1cm} (2)

Where \( i \) varies from 1 to 4 or 1 to 3 when necessary. The optimal number of Maxwell elements was determined using the Chi-square function expressed by equation 3 (typically 3 to 4) and visually matching the modeled stress relaxation curve to the measured curve (figure 1C).

\[ x^2 = \sum_{j=0}^{200} \left( \frac{E_j - E(t_j)}{\sigma_j} \right)^2 \]  \hspace{1cm} (3)

Where \( j \) varies from 0 to 200 seconds, \( E_j \) is the experimentally measured value at time \( j \), \( E(t_j) \) is the fit values at time \( j \) calculated using eq. 1 and \( \sigma_j \) is the standard error which the LLCT makes due to inherent errors in position, time and load measurements.

**Statistical analyzes**

Mechanical characterization measurements were obtained from 3 locations per tissue piece and for each hydrogel 4 replicate gels were made and measured on 3 separate occasions. Data are expressed as median and standard deviation (SD). Statistical analyzes were performed using PRISM 7 software (GraphPAD PRISM, San Diego, CA). Differences between tissue and corresponding ECM hydrogels were tested using Mann-Whitney U test and considered significant when \( p < 0.05 \).

**Results**

**Protein distribution of whole, decellularized and pepsin digested lung tissue**

The banding pattern did not differ between control, COPD GOLD IV and IPF whole tissue (Figure 2A). Decellularized IPF powder had the highest protein content while decellularized control and COPD GOLD IV protein content was hardly detectable by Coomassie staining.

The banding pattern for pepsin digested dECM was similar for all tissue types.
Generation of human lung ECM hydrogels

Pepsin digestion times were varied from 8 to 72 hours (data not shown) and after 72h of digestion, with the addition of NaOH and 10xPBS, a stable hydrogel was generated from control, COPD GOLD IV and IPF pepsin digested dECM.

Human lung ECM hydrogel fiber organization

H&E staining showed a difference in fiber organization between the ECM hydrogels (figure 2B). The IPF ECM hydrogel fibers appeared to form a dense network structure while control and COPD GOLD IV ECM hydrogels formed more open loose structures.

Stiffness of ECM hydrogels resemble native tissue

The stiffness of lung tissue displayed a degree of heterogeneity (Figure 3A), which was most apparent in IPF tissue, ranging from 9 kilopascals (kPa) to 38.5 kPa. Average IPF tissue stiffness (18.9 ± 11.1 kPa) was higher than both control (3.7 ± 1.3 kPa) (p<0.05) and COPD GOLD IV (2.9 ± 0.8 kPa) (p<0.05) lung tissue. dECM hydrogel stiffness followed a similar pattern, with average IPF dECM hydrogel stiffness (6.8 ± 2.8 kPa) also being greater than control (1.1 ± 0.2 kPa) (p<0.05) and COPD GOLD IV (1.5 ± 0.4 kPa) (p<0.05). Each dECM hydrogel had a reduced stiffness compared to their intact tissue counterpart (p<0.05).

Total relaxation of ECM hydrogels does not mimic native tissue

After initial compression of 20%, the dissipation of the force was monitored over 200 seconds (figure 3B). The total stress relaxation of IPF lung tissue was lower (72.1 ± 13.1) than control lung tissue (88.7 ± 10.4 kPa) (p<0.05), which was similar to relaxation of COPD GOLD IV (87.0 ± 7.9 kPa). The total relaxation percentage was 100% for all hydrogels except for IPF lung dECM hydrogels (99.3 ± 0.8 %). The total relaxation for all dECM hydrogels was higher (p<0.05) than that of all corresponding lung tissues.

Maxwell element relaxation time constants (tau) similar between hydrogels and tissue.
The $\tau$'s of all elements for tissue were longer than those of dECM hydrogels ($p<0.05$) (figure 3C). All lung tissues and IPF dECM hydrogels required 4 Maxwell elements to describe the total relaxation seen in figure 3B, while control and COPD GOLD IV dECM hydrogels needed only 3 elements. The $\tau$ of control tissue Maxwell lasted longer than control ECM gels (and required 1 additional element). COPD GOLD IV tissue also required 1 additional element, and each individual element lasted longer than the equivalent element in COPD GOLD IV dECM hydrogel. Lastly, IPF tissue and IPF dECM hydrogel both were described by 4 elements, with the IPF tissue elements lasting longer.

**Maxwell elements relative importance to relaxation of ECM hydrogels and tissue**

The $R_i$ of the 4th Maxwell element described the largest proportion of the relaxation in native lung tissues (control 28.7%, COPD GOLD IV 31.2%), but especially in IPF tissue (44.6%) (figure 3D). In contrast, the $R_i$ of the 1st Maxwell element contributed the most to the relaxation in the dECM hydrogels from all groups (44.6% for control, 44.8% for COPD GOLD IV and 49.7% for IPF). Within the IPF dECM hydrogels a 4th element with a low $R_i$ (10.5%) also contributed to the relaxation. The 2nd and 3rd element contribution was higher in all hydrogels compared to tissue ($p<0.05$) with the exception of the contribution of the 3rd element in IPF tissue and hydrogel which did not differ.

**Discussion**

This study shows, for the first time, that human lung tissue can be decellularized, reduced to a powder and reconstituted as a hydrogel. Furthermore, this can be accomplished with control, COPD GOLD IV and IPF lung tissues, generating a 3D hydrogel that reflects the stiffness of native tissue.

The protein content detected by SDS-PAGE in native lung tissue, decellularized lung and pepsin digested lung dECM were consistent, with the exception of the dECM IPF which was
greater. The native tissues contained total cellular and extracellular components, and given
the equal loading of protein, the expected similarity in protein banding patterns was observed
between control, COPD GOLD IV and IPF. Of the different dECM powders IPF yielded the
highest protein content; however, it was not clear if this reflected a difference in protein yield
or differential solubilization with RIPA buffer that may have been favorable to the proteins
abundant in IPF tissue. Favorably, the protein yield and banding distribution after pepsin
digestion was similar between the different groups.

The 72h pepsin digestion required for generating human lung dECM hydrogels was
substantially longer than that described for decellularized tissue ECMs from other organs
(10). In concert with our findings, Pouliot and colleagues recently described decellularization
and gelation of porcine lung using a pepsin digestion of 42h (22). This difference in required
digestion times may reflect the complexity of the lung matrix.

The measured stiffness of the control and IPF dECM hydrogels resembled the stiffness
previously reported in literature of whole and decellularized human lung samples in these
categories (3). Prior rheological data available on the stiffness of COPD GOLD IV lungs in
literature is limited (16, 27). Herein, the average global stiffness of COPD GOLD IV tissue
was similar to that of control lung tissue.

The dECM hydrogels relaxed completely after compression while tissues did not, irrespective
of the underlying disease. The relaxation behavior of a sample is dictated, in part, by the
topical arrangement of ECM in intact tissue or degree and type (e.g. ionic or covalent) of
internal crosslinking in hydrogels (28, 30). This may explain the higher degree of relaxation
seen in the dECM hydrogels and the reduced degree of relaxation of IPF tissue compared to
COPD GOLD IV and control tissue. The absence of cells in the dECM hydrogels would mean
there were no new covalent crosslinks established within the hydrogels. The greater degree
of matrix organization in the IPF tissue (26) and the stiffer fibroblasts within these tissues
(14) would also contribute to the differences in stress relaxation.
The total relaxation time was longer for lung tissue than for dECM hydrogels, with each Maxwell element contributing to the increased relaxation time. However, the patterns of relative contributions for all the elements were similar between tissue and ECM hydrogels, suggesting that the composition of the ECM in the hydrogels contributes to the relaxation capacity. Linking specific hydrogel components such as water, molecules, cells or ECM to individual Maxwell elements remains difficult such that currently these remain mathematical entities with no clear biological correlations as yet. However, in bacterial biofilms, the constituent components were attributed to Maxwell elements with regards to their contributions to viscoelastic relaxation (21). For dECM hydrogels, the first element made the greatest contribution to the relaxation sequence, possibly reflecting the major role played by the water content of dECM hydrogels and absence of cell-derived or other tissue-related crosslinks. In tissue each element contributed more equally to the relaxation process, except in IPF tissue. The 4th, slowest element made the largest contribution to the relaxation in IPF tissue. Interestingly, all tissues and the IPF ECM hydrogel required 4 Maxwell element models to describe their relaxation while control and COPD GOLD IV dECM hydrogels required 3, further suggesting that the ECM composition also plays a role in viscoelasticity.

Some limitations of our experimental approach must be recognized. Proteoglycans and growth factors were lost or disrupted during the preparation procedure (11, 18) and the influence of these molecules on the rheological properties of the tissues/hydrogels is not known. The approach used herein for measuring the rheology was at a macro (millimeter) scale. How these measurements compare to the nano scale of atomic force microscopy has yet to be examined. All the LLCT measurements were recorded at RT and thus may not fully reflect the biomechanical properties of the lung in vivo.

In conclusion, human lung dECM gels provide new opportunities for simulating the lung microenvironment, enabling the generation of novel models for mimicking native lung ECM in a research environment. Exciting opportunities now exist for exploring the response of human lung derived cells in 3D environments through modulation of parameters including...
stiffness, dimensionality, protein content and protein distribution for ECM from control, COPD GOLD IV and IPF lungs.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

R.H.J.H., M.C.H., M.N.H., and J.K.B conceived and designed research; W.T., classified tissue pathology; R.H.J.H. E.A.G., M.R. and M.R.J performed experiments; R.H.J.H. analyzed data; R.H.J.H., P.K.S., M.C.H., M.N.H., and J.K.B. interpreted results of experiments; R.H.J.H. prepared figures; All authors read and revised the draft manuscript versions; All authors approved the final version.

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**Figure Legends**

Fig. 1. Hydrogel generation and mechanical characterization

_A_: Overview of the decellularization process used for human lung and _B_: the solubilization and gelation process of decellularized human lung. _C_: Low load compression testing measuring stiffness and viscoelastic properties. Samples were compressed by 20% measuring stiffness after which the stress relaxation was monitored as a function of time. Stress relaxation was modeled using generalized Maxwell model with 3-4 elements.

Fig. 2. ECM hydrogel protein distribution and fiber organization

_A_: Protein distribution in intact tissue, decellularized tissue powder and ECM pre-gel for control, COPD GOLD IV and IPF on a 5 & 10% SDS PAGE gel stained with Coomassie brilliant blue. _B_: Hematoxylin & eosin stained sections of control, COPD GOLD IV and IPF dECM Hydrogels at 10x and 20x magnification showing the fiber organization within the ECM hydrogels. Brightness/contrast was adjusted equally for visual presentation of all H&E images.
Figure 3 Stiffness and viscoelasticity of lung tissue and ECM hydrogels

A: The stiffness of native lung tissue and corresponding ECM hydrogels. B: Total relaxation of the compressive force applied at 20% deformation over 200s. C: Maxwell element relaxation time constants. D: The contribution (relative importance) of each Maxwell element to the total relaxation. Measurements were obtained from 3 locations per tissue piece (control n=5, COPD GOLD IV n=5 and IPF n=3) and for each hydrogel 4 replicate gels were made and measured individually on 3 separate occasions. Mann Whitney U test comparing tissue and hydrogel. *p<0.05, **p<0.01, and ***p<0.005.
A

Decellularization

1. Increasing surface area
2. Detergent based decellularization
3. Lyophilizing and milling

B

Hydrogel generation

Decellularized ECM in digestion solution: pepsin in 0.01 HCL for 72h

Digested ECM pre-gel

+ PBS + NaOH + 37°C + 1h

Human lung ECM hydrogel

C

Low load compression Tester (LLCT)

Lung tissue and hydrogel under compression

Stress relaxation under compression

Generalized Maxwell model

Chi-squared evaluation of required Maxwell elements

Modelling of values

Does not fit using 1 Maxwell element

Modelling of values fits using 1 Maxwell element
