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| Advisor 1 Name | Liheng Cai |
| Advisor 2 Name | |
| Committee Member 1 Name | David Green |
| Committee Member 2 Name | Rachel Letteri |
| Committee Member 3 Name | Christopher Highley |
| Committee Member 4 Name | Steven Caliari |
| Committee Member 5 Name | |
| Committee Member 6 Name | |

UPDATE

# A Gel Coated Air-Liquid-Interface Culture System with Tunable Substrate Stiffness Matching Healthy and Diseased Lung Tissues

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A

Thesis

Presented to

the faculty of the School of Engineering and Applied Science

University of Virginia

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in partial fulfillment

of the requirements for the degree

Master of Science

by

Catherine Chu

August 2022

# APPROVAL SHEET

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Author: Catherine Chu

This Thesis has been read and approved by the examing committee:

Advisor: Liheng Cai

Advisor:

Committee Member: David Green

Committee Member: Rachel Letteri

Committee Member: Christopher Highley

Committee Member: Steven Caliari

Committee Member:

Committee Member:

Accepted for the School of Engineering and Applied Science:

Jennifer L. West, School of Engineering and Applied Science

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# Abstract

Since its invention in late 1980s, air-liquid-interface (ALI) culture system has been the golden standard *in vitro* model for studying human airway biology and pulmonary diseases. In a conventional ALI system, however, cells are cultured on a plastic membrane, which is much stiffer than that of human airway tissue. Here, we develop a gel-coated ALI culture system that enables matching the stiffnesses of healthy and fibrotic airway tissues. We determine the optimum gel thickness that does not impair the transport of nutrients and biomolecules essential to cell growth. Similar to the conventional ALI system, the gel-ALI system allows human bronchial epithelial cells to proliferate and differentiate to a pseudostratified epithelium, capturing the essential biological features *in vivo*. We discover that hydrogels of different stiffnesses result in significant changes in morphology and migration of cells, highlighting the importance of the mechanical environment to human airway remodeling. The developed technology should benefit the field of human airway biology and pulmonary diseases.

# Table of content

# Acknowledgement

Foremost, I would like to express my deepest appreciation to my graduate advisor, professor Liheng Cai. I'm extremely grateful for his continuous guidance and advice on my studies that enables me to develop a deeper understanding of the subject. I also wish to thank my lab mate Zhijian He, who has helped me a lot on my research project. Finally, I would like to thank my parents for supporting me unconditionally.

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# Chapter I. Gel-coated Air-liquid Interface (ALI) Cell Culture System

## 1.1 Basic concept of Air-liquid-interface cell culture system

Cell culture model allows for recapitulating essential biological features of human organs or tissues *in vitro*, and thus represents a powerful tool for exploring cell biology, mechanisms of diseases, drug discovery, and tissue engineering. For instance, to study pulmonary diseases, human bronchial epithelial cells are cultured using an Air-liquid-interface (ALI) system to form a pseudostratified epithelium. In a typical culture protocol [1], primary human bronchial epithelial (HBE) cells are seeded onto a porous membrane. Cells initially grow under submerged culture with culture medium supplied to both apical and basal chambers. Once cells reach confluence, the system is transferred to ALI culture by removing cell culture media at the apical compartment. ALI culture initiates cells to differentiate into ciliated cells, goblet cells, and basal cells, eventually forming a pseudostratified columnar epithelium, recapitulating essential biological features of human airway surface *in vivo*. As a result, ALI models are commonly used to investigate chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD), asthma, and cystic fibrosis (CF) [2-3]. Thus far, ALI culture system represents the gold-standard *in vitro* model for studying airway biology and diseases.

A major limitation of classical ALI models is that cells are grown on a porous membrane made of stiff, fragile plastics (e.g., polyester or polyethylene terephthalate (PET)); they have stiffness on the order of GPa, more than $10^5$ times stiffer than human airway tissues [4]. In the human airway, epithelial cells are grown on the soft lung extracellular matrix (ECM) that provides structural support and mechanical stability to the airway epithelium, as illustrated in Figure 1.1 (a).

The mechanical properties of ECM play a crucial role in influencing airway cell behavior and function [5-7]. For example, ECM mimicking substrate stiffness affects the morphology of alveolar epithelial cells along with the size of focal adhesions and the actin cytoskeleton [8]. Another drawback of conventional ALI model is that the plastic membrane has a fixed thickness and cannot be adjusted. However, the stiffness of the lung ECM varies with pulmonary injuries or diseases. For example, idiopathic pulmonary fibrosis (IPF) is a progressive lung disease of unknown cause; yet it is associated with dysregulated lung remodeling that produces massive ECM deposition in lung, leading to tremendously high lung stiffness compared to healthy lung tissue. Consequently, it is highly needed for an in vitro culture system integrated with an ECM mimicking matrix to provide a platform with adjustable stiffness for epithelial cell culture.

**1.2 Design of gel-coated ALI culture system**

To further improve the ability for basal cells to differentiate into mature lung phenotypes and form bronchial epithelium, three-dimensional (3D) cell cultures are developed. 3D cell models integrate a new material to represent the interstitial matrix. A variety of materials have been used to substitute or coat the plastic membrane in the ALI culture system. Naturally derived ECM materials, such as collagen, alginate [9], elastin [10] and Matrigel [11], can better mimic the biophysical and biochemical properties of ECM. By contrast, synthetic ECM materials can precisely control the stiffness. Polydimethylsiloxane (PDMS) [12-13] is commonly used as a porous membrane due to its high elasticity, opacity, and biocompatibility. Although the stretchable PDMS membrane has been often integrated into microfluidic devices to mimic the cyclic mechanical strain of the lung alveolar barrier, PDMS absorbs hydrophobic small molecules and drugs which may affect the results of drug testing [14-15]. Another synthetic material as the ECM substitute is polyacrylamide (PAAm) hydrogel. Polyacrylamide hydrogel is well adapted to *in vitro* cell culture

because PAAm hydrogel is bioinert and can be easily synthesized. Moreover, the stiffness of PAAm hydrogel can be controlled by adjusting the monomer and crosslinker concentrations. A magnetic microboat device integrating with PAAm hydrogel has been developed to mimic the ECM of the healthy lung and the lung with cystic fibrosis [16]. Yet, the PAAm gel thickness in the magnetic microboat is 1 mm thick, which might prolong the time for nutrients and biologically important molecules to transport. Moreover, the complexity of magnetic microboat requires manual operations. To circumvent these shortcomings, we aim to develop an easy-to-use cell culture system that controls the substrate stiffness based on standardized ALI culture system.

Here, we design an *in vitro* culture system that integrates a thin layer of polyacrylamide hydrogel with the ALI culture system, as schematically illustrated in Figure 1.1 (b). In the PAAm gel-ALI culture system, Transwell® permeable supports are inserted in the multiple-well plate and immersed by cell culture media. The soft PAAm hydrogel layers are fabricated on the porous membrane, and then cells are seeded on the hydrogel layers. On top of the PAAm gel, we design to coat the hydrogel with sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (also known as sulfo-SANPAH) and type I collagen which promotes the cells to attach to the hydrogel. The stiffness of polyacrylamide hydrogel can be easily controlled to match the ECM stiffness in healthy and diseased lungs, ranging from 0.25 kPa to 50 kPa, by tuning the monomer and crosslinker concentration.

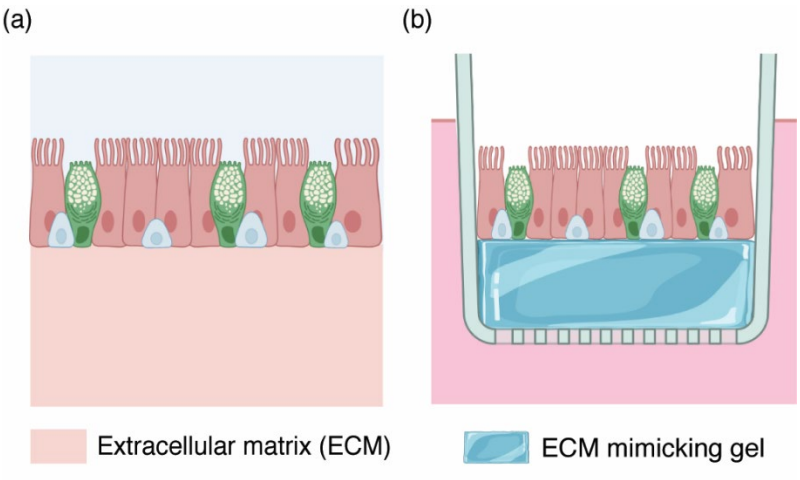


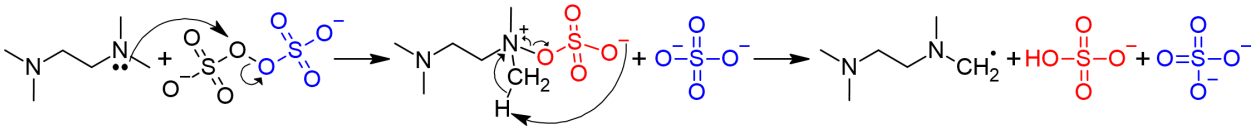

**Figure 1.1** Illustration of Gel coated Air-Liquid Interface (gel-ALI) culture system. (a) In human airway, epithelial cells are supported by soft extracellular matrix (ECM). (b) In gel-ALI culture system, epithelial cells are cultured on a thin layer of hydrogel with stiffness matching that of the ECM in health and disease.

# Chapter II. Physical properties of polyacrylamide hydrogel

## 2.1 Synthesis of polyacrylamide hydrogel

We use polyacrylamide hydrogel, one of the primarily used hydrogels as substrates to simulate the physical properties of ECM, to mimic the mechanical properties of human lung tissue. PAAm hydrogel can be easily synthesized using a one-step free radical polymerization. Moreover, the gel surface can be chemically modified to link with protein crosslinkers, which can be used to conjugate with ECM proteins and further promote cell attachment.

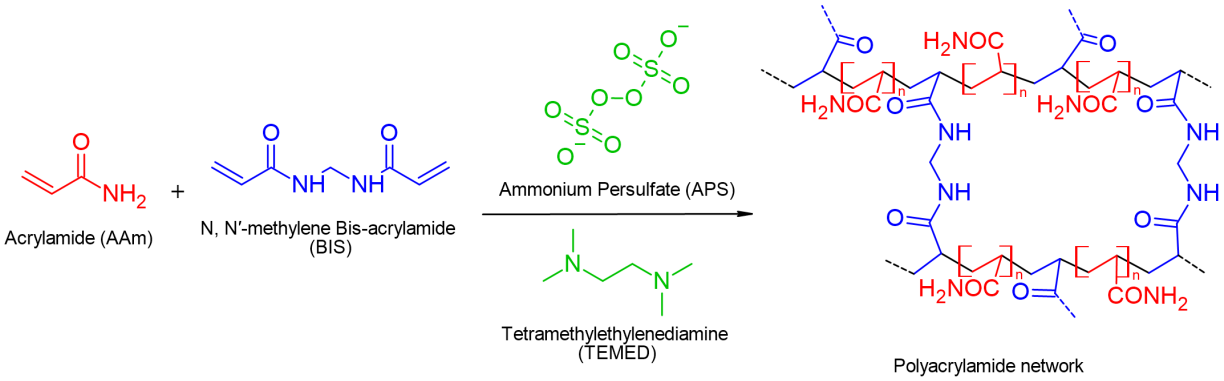
The standard free radical polymerization reaction of polyacrylamide hydrogel consists of four components: acrylamide (AAm) monomer, N, N′-methylene bis-acrylamide (BIS) crosslinker, ammonium persulfate (APS) initiator, and tetramethylethylenediamide (TEMED) initiator. Combining APS with TEMED (Scheme 2.1), the long pair electrons of nitrogen atoms in TEMED attack the oxygen atom of APS to form an N-O bond and break the O-O bond in APS [17]. This creates a TEMED-sulfate intermediate and a sulfate ion. Then the oxygen anion of the TEMED-sulfate intermediate extracts a hydrogel from the dimethyl group and turns into a TEMED radical ion and another radical sulfate ion. TEMED can not only act as an initiator to start the polymerization but also act as a catalyst to speed up the polymerization process at low temperatures.



**Scheme 2.1** Initiation mechanism of TEMED and APS.

Consequently, the free radicals begin the polymerization by breaking the double bonds (vinyl groups) of acrylamide and converting acrylamide monomers into free radicals (Scheme 2.2). Then the acrylamide radicals react with inactivated monomers to start the polymerization chain

reaction. The polymer chains are randomly crosslinked with bis-acrylamide and eventually form polyacrylamide hydrogel.




**Scheme 2.2** Polymerization of polyacrylamide hydrogel.

To synthesize the polyacrylamide hydrogel, we first prepare a polyacrylamide gel precursor solution, and then use APS as an initiator to activate polymerization (Figure 2.1). We prepare a polyacrylamide gel precursor solution by mixing 200 µL 40% acrylamide solution, 200 µL 2% bis-acrylamide solution, and 776 µL sterile de-ionized water. Then, we add 12 µL TEMED solution to the gel mixtures to achieve a final TEMED concentration of 0.012%. Afterward, 12 µL APS is added to reach a final APS concentration of 0.0012%. We quickly apply 30 µL of the 1 mL reaction mixture to the center of each Transwell insert and cover the solution with an 11 mm circular coverslip. The coverslip is custom-made by punching an 11 mm circular disk made of three layers of Scotch® tape. The coverslip prevents the gel mixture from contacting oxygen, which is known to inhibit the polymerization of the solutions. After 30 minutes, we remove the coverslip, seal the 12-well culture plate, and keep the plate at 4 ºC overnight to completely polymerize the hydrogel.

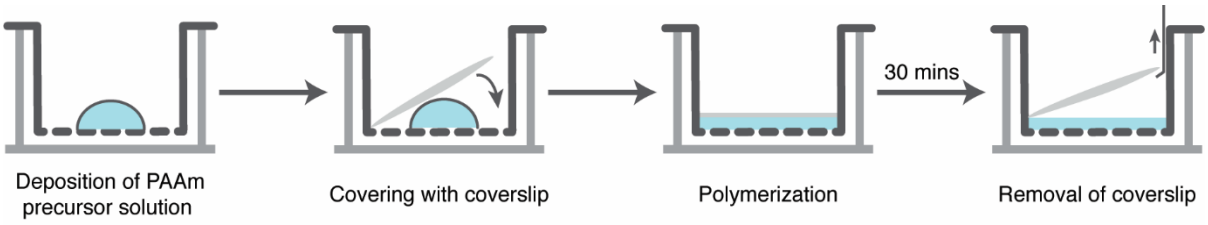



**Figure 2.1** Synthesis of polyacrylamide hydrogel.

## 2.2 Mechanical properties of polyacrylamide hydrogel

The mechanical properties of the ECM influence the regulation of cell behavior. Therefore, the matrix elasticity of polyacrylamide gel as the ECM mimicking substrate is a necessary experimental parameter for *in vitro* 3D cell cultures. To synthesize polyacrylamide gel as ECM mimicking substrate, we first determine the stiffness of PAAm hydrogel that match the stiffness of biological tissue, including healthy airway tissue and IPF airway tissue.

To characterize the matrix elasticity of polyacrylamide hydrogel, we first assume that PAAm gel adheres to the affine network model. The affine network assumes that the relative deformation of each individual polymer strand equals the macroscopic deformation of the whole network. The stiffness of the PAAm hydrogel can be expressed with Young's modulus and calculated by the following formula [18-19]:

$$E = 2G(1 + \nu)$$

where E is the Young's modulus, G is the shear modulus, and ν is the Poisson's ratio. The Poisson's ratio of 0.5 was employed for the calculation of Young's modulus, as Poisson's ratio of rubberlike materials such as hydrogels is mostly close to 0.5 based on previous studies [20]. Therefore, Young's modulus of polymer can be estimated as three times of the shear modulus. Furthermore, shear modulus is defined as the ratio of shear stress to the shear strain. To measure the shear stress and shear strain of the PAAm hydrogel, we conduct experiments using rheometer.

We conduct a frequency sweep to characterize the relationship between polyacrylamide hydrogel stiffness and monomer concentration. We prepare samples with acrylamide and bis-acrylamide 7.5%, 10%, 12.5%, 15%, 17.5%, 20% and 22.5% (Table 2.1), respectively. We add DI water, TEMED, and APS to the precursor solution. We quickly apply 800 μL of gel mixture to the 12 well culture plate and cover the solution with 21 mm circular glass coverslips. After 30 minutes, we remove the coverslips and perform rheological measurements. Rheological measurements are conducted using a stress-controlled rheometer (Anton Paar, MCR 302) with a plate-plate geometry of diameter 8 mm. We measure the storage modulus at the shear strain of 0.5 % and the shear frequency varying from 0.1 rad/sec to 100 rad/sec. Measurements for each group were performed three times.

**Table 2.1** Composition of reactants for the synthesis of PAAm gel samples.

| Sample | 40% AAm (μL) | 2% BIS (μL) | TEMED (μL) | 10% APS (μL) | $H_2O$ (μL) |
|---|---|---|---|---|---|
| AAm/BIS 7.5% | 75 | 75 | 12 | 12 | 826 |
| AAm/BIS 10% | 100 | 100 | 12 | 12 | 776 |
| AAm/BIS 12.5% | 125 | 125 | 12 | 12 | 726 |
| AAm/BIS 15% | 150 | 150 | 12 | 12 | 676 |
| AAm/BIS 17.5% | 175 | 175 | 12 | 12 | 626 |
| AAm/BIS 20% | 200 | 200 | 12 | 12 | 576 |
| AAm/BIS 22.5% | 225 | 225 | 12 | 12 | 526 |

To determine the stiffness of the PAAm hydrogels that match healthy and IPF lung tissues, we measure the storage modulus of PAAm hydrogel under different AAm and BIS concentrations. The storage modulus increases when the AAm and BIS concentration increases, as shown in Figure 2.2. Quantitatively, the storage modulus of AAm/BIS 10% gel is about 1.1 kPa and the storage modulus is around 15.7 kPa for AAm/BIS 20% gel (Table 2.2). As recent studies estimate, Young's modulus ranges from 0.5 to 5 kPa for healthy lung tissue, whereas IPF lung tissue has a

high stiffness of 15 to 100 kPa [21-23]. Based on the data, we use PAAm hydrogel made from 10% AAm/BIS with the stiffness of 3.4 kPa for mimicking the stiffness of healthy lung tissue, and PAAm AAm/BIS 20% gel with the stiffness of 47.1 kPa for mimicking IPF lung tissue. These results demonstrate that the stiffness of polyacrylamide hydrogel can be precisely controlled by the concentration of acrylamide and bis-acrylamide. This feature allows us to synthesize PAAm hydrogel that mimics the stiffness of biological tissues.

**Table 2.2** Relationship between AAm/BIS concentration and storage modulus.

| Sample | Storage modulus (Pa) | Estimated Young's modulus (Pa) |
|---|---|---|
| AAm/BIS 7.5% | 84.82 | 254.46 |
| AAm/BIS 10% | 1139.55 | 3418.65 |
| AAm/BIS 12.5% | 2421.33 | 7264 |
| AAm/BIS 15% | 5500.7 | 16502.1 |
| AAm/BIS 17.5% | 10203.83 | 30611.5 |
| AAm/BIS 20% | 15705.33 | 47116 |
| AAm/BIS 22.5% | 20842.33 | 62527 |

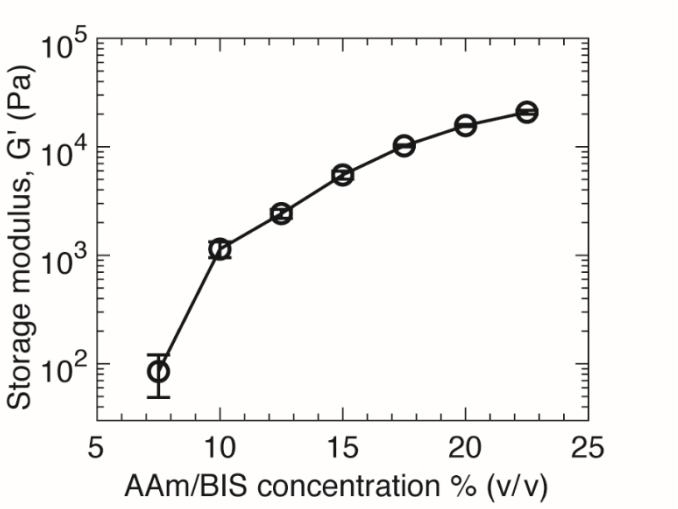



**Figure 2.2** Dependence of the storage modulus of the PAAm hydrogel under different AAm/BIS concentration measured at 20 ºC at a fixed strain of 0.5% (n = 3).

### 2.3 Mesh size of polyacrylamide hydrogel

PAAm hydrogel consists of long polymer chains that are chemically crosslinked by acrylamide and bis-acrylamide to generate a three-dimensional network (Figure 2.3). In addition to polymer networks and crosslinks, hydrogels have vacancies that allow substances to pass through. Mesh size (ξ) is the linear distance between two adjacent crosslinks.

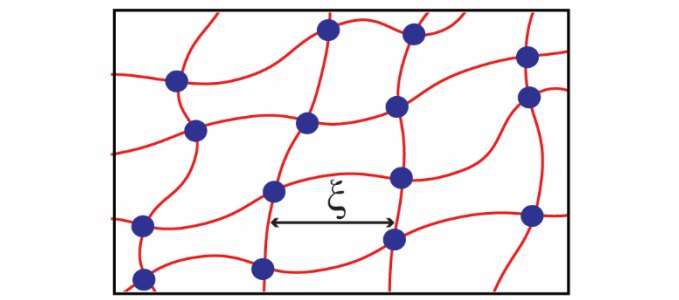



**Figure 2.3** PAAm network (blue dots: bis-acrylamide crosslinker, red lines: acrylamide chains).

The theoretical prediction of mesh size of hydrogel network can be estimated based on the equation:

$$\xi \approx (\frac{k_B T}{G})^{\frac{1}{3}}$$

where G is shear modulus, $k_B$ is the Boltzmann constant and T is the absolute temperature [19]. Shear modulus equals to shear stress divided by shear strain and can be obtained from rheology test. The shear modulus can be quantified by previous rheological measurement (Figure 2.2), which is 1.2 and 15.3 kPa for AAm/BIS 10% and 20 % gel, respectively. Therefore, the mesh sizes of PAAm hydrogel with AAm/BIS 10% and 20% are estimated to be 15.04 nm and 6.416 nm, respectively.

Table 2.3 Rheological parameters of PAAm hydrogel.

| Sample | Shear stress (Pa) | Shear strain (%) | Shear modulus (Pa) |
|---|---|---|---|
| AAm/BIS 10% | 6.068 | 0.502 | 1208.752 |
| AAm/BIS 20% | 78.159 | 0.502 | 15569.588 |

ALI cell culture medium is used to support cell differentiation on porous structure. ALI medium is comprised of glycoproteins, vitamins, inorganic salts, growth factors, hormones, antibiotics, and trace elements that provide nutrients to the cells. One biggest molecule in the ALI medium is holo-transferrin (76 – 81 kDa), whose minimal radius of a sphere protein molecule is 2.4 nm to 3.05 nm. The obtained mesh size for PAAm hydrogel with AAm/BIS 10% and 20% are larger than the diameter of holo-transferrin. Therefore, nutrients in the ALI medium can

theoretically pass through the PAAm hydrogel and eventually arrive at the gel surface where cells can absorb them. To further monitor the diffusion behavior of nutrients, a trace amount of Texas Red dextran with an average molecular weight of 70 kDa was added to the PAAm hydrogel to simulate the nutrient movement. The Texas Red dextran has a Stoke-Einstein radius larger than 6.4 nm [24], which is larger than the size of protein molecules in the ALI culture medium [25]. The diffusivity of dextran will indicate whether the nutrients in ALI culture medium can nourish HBE cells on the PAAm gel surface or not.

**2.4 Permeability of polyacrylamide hydrogel**

Permeability of the hydrogel layer determines its ability to exchange molecules between the basal and apical chamber in the ALI culture system. In gel-coated ALI culture, cells are cultured on the PAAm gel surface, whereas the cell culture media is added to the basal side of the Transwell inserts. Therefore, we test whether the nutrients and biomolecules in cell culture media can diffuse through the polyacrylamide hydrogel and reach the cells in the gel-ALI culture system.

To characterize the permeability of pure PAAm hydrogel, we add fluorescent dye to the PAAm hydrogel and quantify fluorescent intensity through time using a confocal microscope (Leica SP8) (Figure 2.4). We use Invitrogen dextran (Texas Red, MW 70,000 Da) as the fluorescent-labeled molecule to simulate the diffusion of large particles. For monitoring pure PAAm hydrogel (Figure 2.4), we mix fluorescent-labeled dextran with precursor gel solution (AAm/BIS 10 % and 20 %) and synthesize PAAm hydrogel using the same protocol described above. We add DI water at the apical side of the transwell to immerse the gel and observe the change of fluorescent intensity. We acquire cross-section (xz scanning mode) images of PAAm hydrogels using a 10x objective, along with a 488 nm diode laser to excite FITC. The fluorescence

emission is collected by a PMT detector with the peak wavelength between 494 and 520 nm. We quantify and normalize the fluorescence intensity to the initial time point using Fiji.

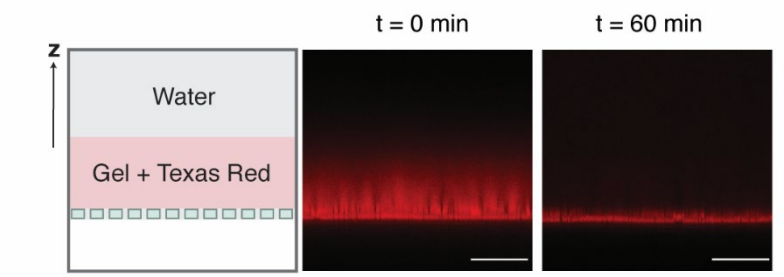



**Figure 2.4** Fluorescence confocal microscopy images of dextran release from 100 μm PAAm hydrogel to water in apical chamber. Scale bar, 100 μm.

### 2.4.1 Dependence of permeability on gel thickness

To determine the relationship between hydrogel thickness and its diffuse properties, we synthesize AAm/BIS 20% PAAm hydrogel with different thicknesses and quantify the fluorescence intensity for one-hour diffusion. For pure PAAm hydrogel, the diffusion of dextran particles is prolonged as the hydrogel thickness increases from 100 μm to 1000 μm (Figure 2.5), meaning it takes longer for dextran to diffuse in the thicker hydrogel. The fluorescent intensity of 100 μm hydrogel reaches its half-decay time at 12 minutes, and then decays to 25 % of its initial intensity at one hour. By contrast, 1000 μm hydrogel shows a negligible decrease in intensity, as the intensity decreases to 75% of its initial intensity after one hour. This result indicates that the diffusion of large particles in the hydrogel is determined by the gel thickness. Therefore, we apply PAAm hydrogel with 100 μm thickness in the gel-ALI culture system to avoid the thickness effect on the diffusion of cell culture media.

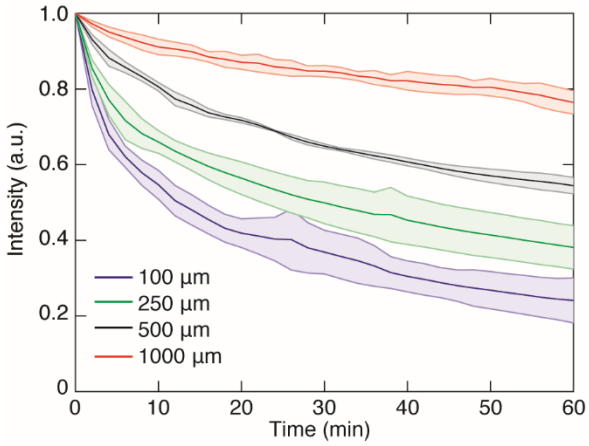



**Figure 2.5** Dependence of fluorescence intensity on diffusion time under different gel thickness. The error bar is the standard deviation of measurements from different hydrogel samples (n = 3).

### 2.4.2 Dependence of permeability on gel stiffness

To determine the relationship between hydrogel stiffness and its diffuse properties, we synthesize 100 µm thickness PAAm hydrogel with different stiffness (AAm/BIS 10 % and 20 %) and quantify the fluorescence intensity for one-hour diffusion (Figure 2.6). For pure PAAm hydrogel, it takes longer for dextran to diffuse as the acrylamide and bis-acrylamide concentration increases from 10 % to 20%. Quantitatively, the half-decay time of fluorescent intensity increases from 5 to 12 minutes. As established in the prior calculation, the mesh size is estimated to be 15 nm and 6.4 nm for AAm/BIS 10% and 20 % gel, respectively. These results prove that hydrogel stiffness is determined by the mesh size of the hydrogel network and results in different diffuse times for dextran diffusion. Yet, the standard deviation error bars of AAm/BIS 10% and 20 % gel are mostly overlapped after the first 30 minutes, suggesting that the difference between the two curves is not significant. This is likely because fluorescent particles in a thin hydrogel layer (thickness 100 µm) can easily escape the hydrogel network, resulting in a slight diffusion difference of different stiffness hydrogels.

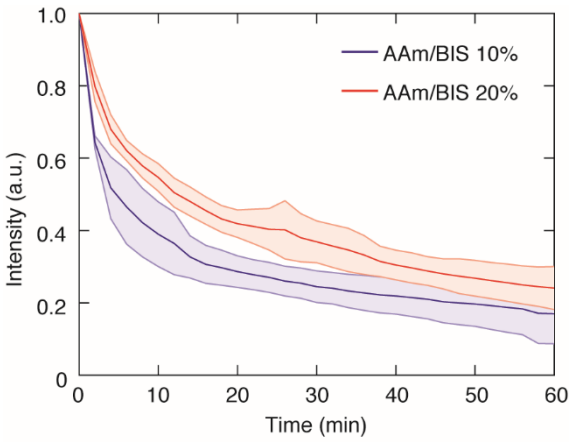



**Figure 2.6** Dependence of fluorescence intensity on diffusion time under different gel stiffness. The error bar is the standard deviation of measurements from different hydrogel samples (n = 3).

### 2.4.3 Permeability of PAAm gel in gel-coated ALI system

To determine the permeability of PAAm gel on the Transwell insert, we add the fluorescent-labeled dextran to the water on the apical side, as shown in Figure 2.7. We quantify the diffusion profile of dextran molecules by setting 25 μm above the boundary of membrane and hydrogel as z = 0. We normalize the intensity to the maximum intensity of the diffusion curve.

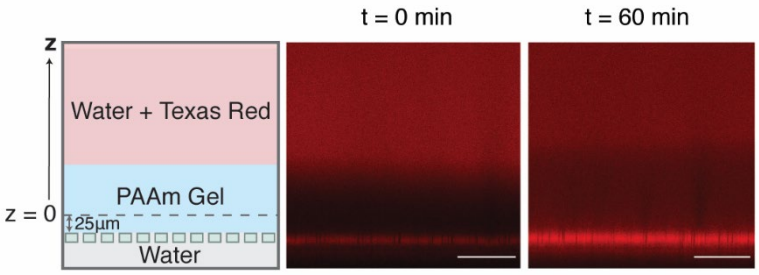



**Figure 2.7** Fluorescence confocal microscopy images of dextran release from water in the apical chamber to AAm/BIS 10% gel from t = 0 to t = 60 minutes. Scale bar, 100 μm.

This result shows that most dextran molecules can diffuse from the water in the apical chamber to the PAAm gel in the first 20 minutes, and the fluorescent intensity gradually reaches equilibrium after 40 minutes (Figure 2.8 (a)). Moreover, the dextran molecules diffuse faster in

AAm/BIS 10% gel compared to AAm/BIS 20% gel. We further quantify the fluorescent intensity of the gel area from z = 0 to z = 20 μm, which is the farthest place from the dextran particles in the PAAm gel, as shown in Figure 2.8 (b). The intensity exhibits a sharp increase to 50% of its maximum intensity at 6.5 and 9 mins for AAm/BIS 10% and 20% gel. These results show that the diffusion of large particles in the hydrogel is determined by the gel stiffness, yet the dextran diffusion time in AAm/BIS 10% and 20% gel is not significant due to the thin thickness. This validates that the diffusion properties of PAAm hydrogel remain similar when applying the PAAm gel to the Transwell inserts in the ALI culture system.

Collectively, our results demonstrate that thin gel allows dextran particles to transport through a short time and the diffusion has no significant difference under different stiffness, suggesting the gel-ALI culture system does not influence the nutrient supply to the cells.

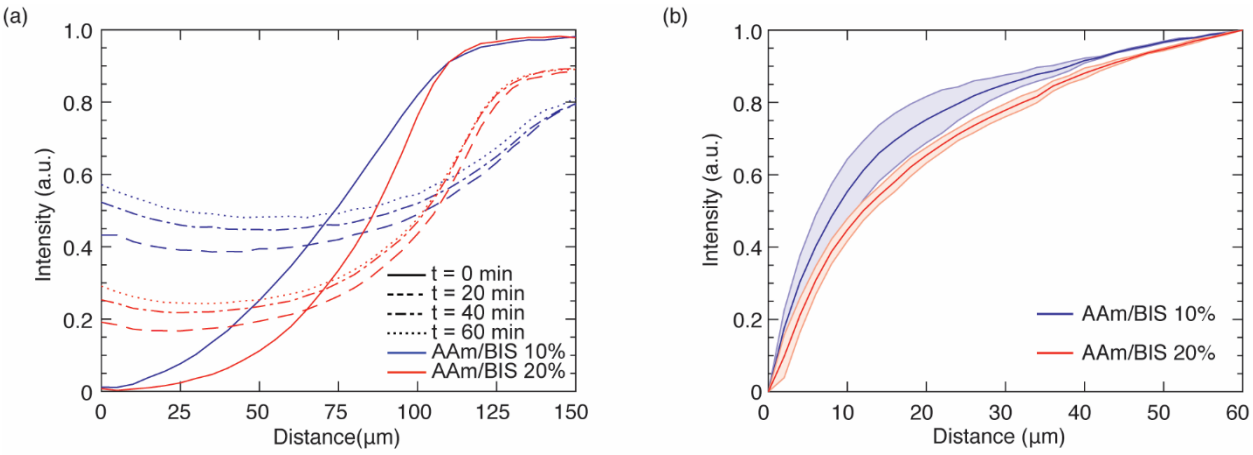


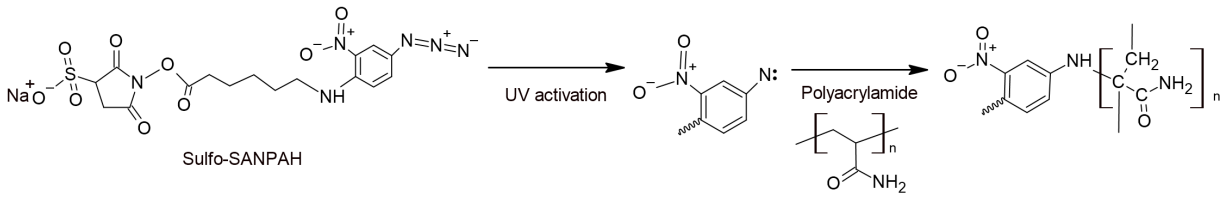

**Figure 2.8** (a) Diffusion as a function of distance from the 100 μm gel above the Transwell membrane to the apical chamber under different gel stiffness. (b) Dependence of fluorescence intensity on diffusion time of z = 0 to z = 20 μm under different gel stiffness. The error bar is the standard deviation of measurements from different hydrogel samples (n = 3).

# Chapter III. Gel-coated ALI System for Human Bronchial Epithelial Cell (HBECs) Culture

## 3.1 Fabrication method of gel-coated ALI system

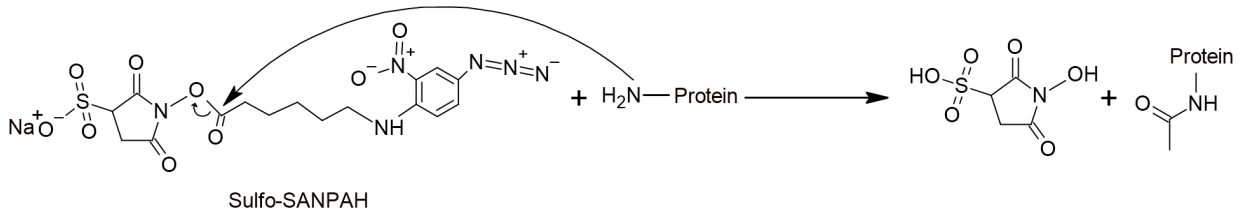
The gel-ALI culture system consists of three moduli: (1) a Transwell insert with a permeable porous membrane, (2) a thin layer of polyacrylamide hydrogel on the membrane, and (3) a coating layer of type I collagen on top of the PAAm gel. Although the adhesion of epithelial cells is weaker on soft hydrogel, we manage to coat the gel with an additional layer of protein crosslinker which promotes the cells to attach to the hydrogel.

Polyacrylamide hydrogel does not allow easy protein attachment. To connect PAAm hydrogel with type I collagen, we use the photoactivable heterobifunctional crosslinker sulfo-SANPAH. Sulfo-SANPAH has amine-reactive N-hydroxysuccinimide (NHS) ester at one end and photoactivatable nitrophenyl azide at another end (Scheme 3.1). When exposed to UV light, the nitrophenyl azide group forms a nitrene group that reacts with the C-H bond in the polyacrylamide backbone.




**Scheme 3.1** Reaction of sulfo-SANPAH with polyacrylamide hydrogel.

On the other end, sulfo-NHS ester reacts with primary amine of collagen and form a stable conjugate, as illustrate in Scheme 3.2. This process successfully conjugate polyacrylamide hydrogel with proteins, and further enhance the attachment of HBE cells on the PAAm gel.




**Scheme 3.2** Reaction of sulfo-SANPAH with protein.

After fabricating PAAm gel on the Transwell insert, we activate the gel surface using sulfo-SANPAH and then coat the gel with type I collagen to promote the attachment of HBE cells to the hydrogel surface (Figure 3.1). We dissolve sulfo-SANPAH in dimethylsulfoxane (DMSO) at 50 mg/mL. Then, we add 100 μL sulfo-SANPAH solution onto the gel surface. Afterward, the gel is exposed to UV light (wavelength 365 nm, intensity 37 mW) for 5 mins. After photoactivation, sulfo-SANPAH is attached to the hydrogel and is ready to react with type I collagen. We rinse the gel using DI water three times and then immerse the gel in DI water at 4 ºC overnight to remove any unreacted sulfo-SANPAH. Next, we apply 0.5 mL of the type I collagen solution (Purecol, 0.2 mg/mL) to the sulfo-SANPAH-coated gel at 4 ºC overnight. On the next day, we rinse the gel with DI water and PBS solution three times to remove excess collagen solution.

After coating the PAAm gel with type I collagen, we sterilize the gel under UV light for 15 minutes and seed the HBE cells onto the gel-coated Transwell inserts. The process of culturing HBE cells from monolayer basal cells to fully functioning bronchial epithelium contains two necessary parts: expansion submerge culture and differentiation ALI culture. Firstly, HBE cells are grown in 500 μL PheumaCult-Ex plus basal medium supplemented with 0.1% heparin in an incubator at 37 ºC and 5% $CO_2$. We also add 1 ml basal media to the basal chamber. The basal medium is changed every other day until cells are 80 ~ 90% confluent. Once the HBE cells in submerge culture reach confluence, we air-lift the system to create an air-liquid-interface. We

remove PheumaCult-Ex plus basal medium from both apical and basal compartments. Then, we add 1 mL PneumaCult ALI medium to the basal chamber and keep the apical chamber dry.

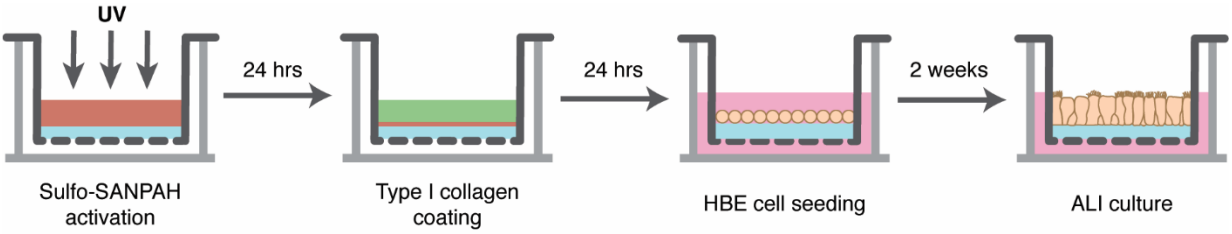



Figure 3.1 Coating process of sulfo-SANPAH and type I collagen on PAAm hydrogel.

### 3.2 HBECs immunofluorescence staining

Airway pseudostratified epithelium consists of various types of cells, including basal cells, ciliated cells, and goblet cells [26]. Basal cells can differentiate into other types of cells found in the epithelium, and even dedifferentiate into stem cells to help repairing airway epithelium [27]. Ciliated cells have beating cilia that physically remove respiratory pathogens and inhaled particles. Goblet cells are accountable for secreting mucus that helps maintain moisture and trap foreign insults. When culturing HBE cells at the air-liquid-interface, undifferentiated basal cells can differentiate into ciliated cells and goblet cells to form a pseudostratified epithelium. Therefore, we investigate whether gel stiffness affects basal cell differentiation.

To visualize the differentiation of airway epithelial cells in the gel-ALI culture system, we perform immunofluorescence staining to observe HBE cells on Transwell inserts without gel coating, PAAm AAm/BIS 10% gel, and PAAm AAm/BIS 20% gel after four weeks of ALI culture. To compare HBE cells in conventional ALI culture and PAAm gel-ALI culture, we culture cells on the Transwell membrane treated with type I collagen.

Immunostaining is a standard method to detect antigens (proteins) in cells based on the specific binding of an antibody to an antigen. This process involves using a primary antibody that targets to react with the antigen on cells. Then a second antibody is applied to bind to the primary antibody as well as helping to amplify the detection signal. The secondary antibody is also conjugated with the fluorescent marker and can be visualized using a fluorescence microscope.

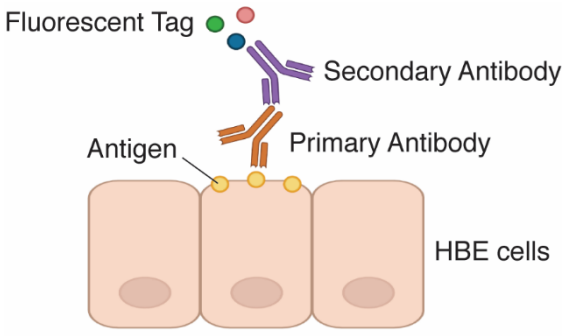



**Figure 3.2** Indirect method of immunohistochemistry.

We conduct immunostaining by first immersing the cells with paraformaldehyde (PFA, 4%) for 15 mins to fix the cells and maintain their structure, and then we add dithiothreitol (DTT, 10mM) to treat cells for 15 mins. Afterward, the cells are immersed in permeabilizing solution (0.2% Triton X-100 in PBS). Then, we block the cells using 3% BSA for one hour to prevent non-specific staining of antibodies. We remove the blocking buffer and incubate cells with rat anti-alpha tubulin primary antibody 1:250 dilution in 3% BSA and mouse anti mucin 5AC (MUC5AC) primary antibody 1:1000 dilution in 3% BSA overnight at 4 ºC. Anti-acetylated tubulin antibodies are applied to label ciliated cells and anti-MUC5AC antibodies are used to mark goblet cells. After washing the cells with PBS for 7 mins three times, we stain cells with secondary antibody solution in 3% BSA for one hour. Finally, we incubate cells with 4’,6-diamidino-2-phenylindole (DAPI) 1:1000 solution in sterilized DI water for 5 mins to stain the nuclei. The cells are visualized by confocal microscope.

Basal cells growing on the Transwell inserts in conventional ALI culture are fully differentiated into pseudostratified epithelium with ciliated cells (red fluorescence in Figure 3.3 (a)) and goblet cells (green fluorescence in Figure 3.3 (a)). Basal cells growing on PAAm AAm/BIS 10% (Figure 3.3 (b)) and 20% (Figure 3.3 (c)) gel are also capable of differentiating into ciliated cells and goblet cells. The cell survival indicates the transport of nutrients in cell culture media is sufficient to transport through the PAAm hydrogel and reach the cells. Although HBE cells in gel-ALI culture differentiate into goblet cells and ciliated cells, the amount of goblet cells is less when culturing on PAAm gel with 20% AAm/BIS, compared to the cells culturing on the plastic membrane. This suggests that substrate stiffness might affect cell behaviors.

Based on our observation, the gel-ALI culture system is proved to be cytocompatibility for HBECs growth and differentiation. To further investigate the impact of ECM-mimicking substrate stiffness on HBE cells, we observe HBE cells under different gel stiffnesses by quantifying cell density, cell migration, and cell shape index.

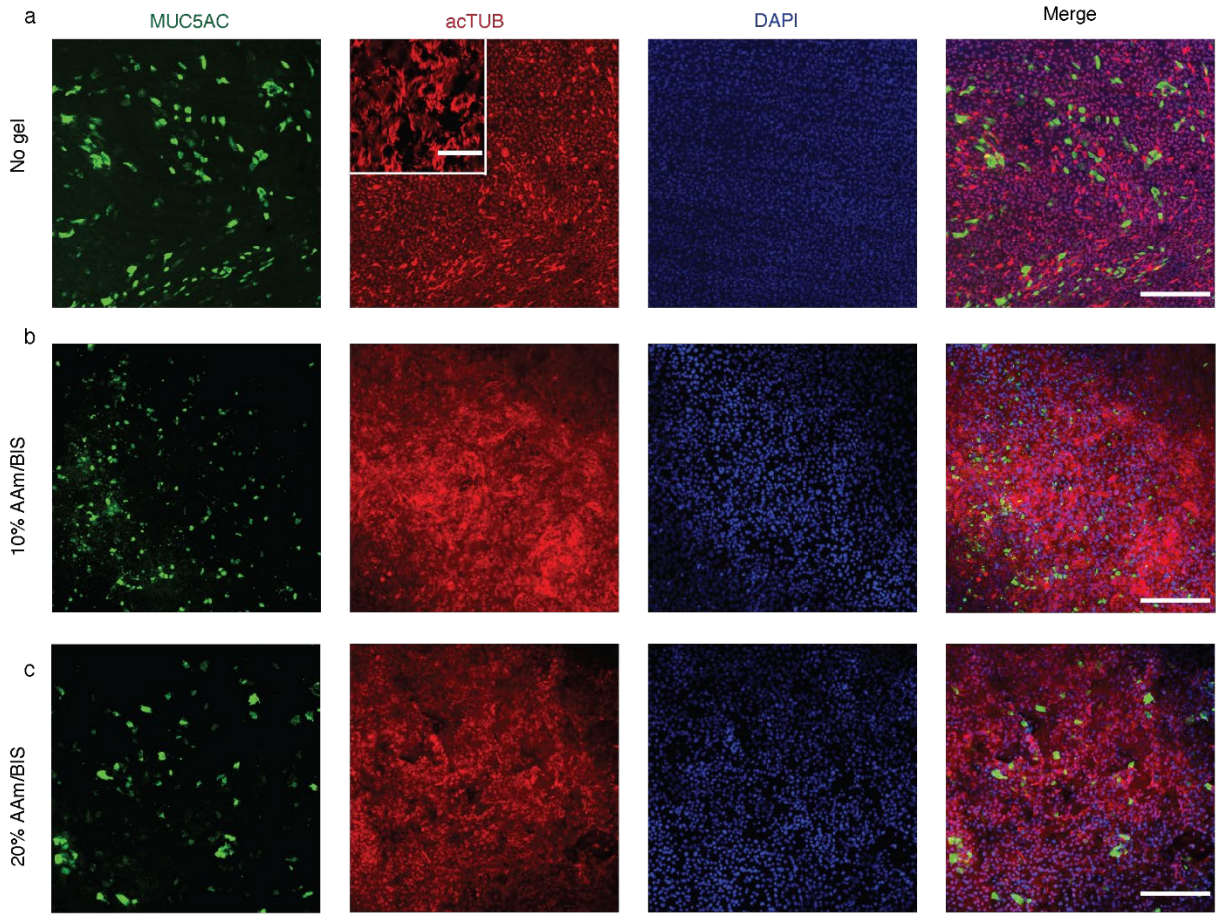



**Figure 3.3** Immunostaining of HBE cells on (a) conventional ALI culture without gel coating (control group), (b) gel-ALI culture on AAm/BIS 10% PAAm hydrogel and (c) gel-ALI culture on AAm/BIS 20% PAAm hydrogel after four weeks of ALI culture. The goblet cells, ciliated cells, and basal cells were revealed by their specific markers: MUC5AC, tubulin, and DAPI. Scale bar: 300 μm. Scale bar in the inset: 20 μm.

### 3.3 Cell density of HBE cells in PAAm gel-ALI system

Cell density has been an indicator of cell responses to different biophysical cues. An abnormal increase or decrease of cell density can reflect the inability of the cells to accommodate cell proliferation or differentiation. Average cell size can also be used to observe cell behavior since the increased cell density associates with the decrease in average cell size.

To study the response of HBE cells to substrate stiffness, we culture HBE cells on Transwell membrane, AAm/BIS 10% PAAm gel which is equivalent to the stiffness of lung tissue, and AAm/BIS 20% PAAm gel that mimic the stiffness of IPF lung. We obtain bright field images of HBE cells in the gel-ALI culture system with different gel stiffnesses using the phase-contrast mode of confocal microscopy on ALI day 7, 14, and 21. We randomly take 4 images from each well, 3 wells in total. We measure cell density by choosing 3 random areas in cell images and then quantify the cell amounts.

The results show that HBE cells cultured on plastic membrane have the highest cell density compared to cells growing on a soft PAAm hydrogel layer after two weeks of ALI culture (Figure 3.4). Likewise, the confocal images confirm that HBE cells are larger when growing on the Transwell membrane, as shown in Figure 3.5 (a). Moreover, the density of HBE cells cultured on AAm/BIS 20% gel is larger than the density of cells on AAm/BIS 10% gel. This might suggest that lower substrate stiffness slows down cell proliferation. Therefore, the results demonstrate that substrate stiffness affects cell density and HBE cells have lower cell density when culturing on the softer substrate. Likewise, the quantification of the average cell area demonstrates this result (Figure S2).

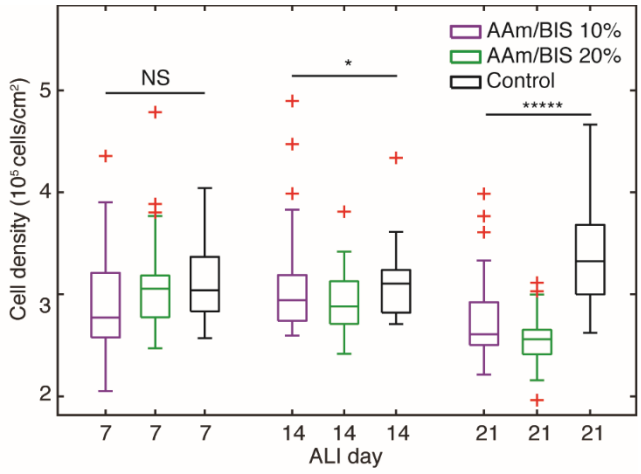



**Figure 3.4** The cell density of HBE cells under different substrate stiffness on ALI culture day 7, 14 and 21. * $p < 0.1$, ***** $p < 0.00001$. Data is analyzed by one-way analysis of variance (ANOVA).

### 3.4 Cell migration speed of HBE cells in PAAm gel-ALI system

Cell migration is a typical process of cell development and maintenance. Cells often migrate in specific directions when that area needs to differentiate cells to heal a wound or induce immune responses. Moreover, cell migration may differ with external signals, such as matrix stiffness [28].

We monitor HBE cell migration with a confocal microscope equipped with a cell culture incubator (37 ºC and 5% $CO_2$). We monitor cell migration under different substrate stiffnesses on ALI day 7, 14, and 21. We acquire time-lapse cell images every 2 minutes with a total of 100 frames. We compute the cell migration speed and generate migration speed maps using the particle image velocimetry (PIVlab) software [29].

We investigate cell migration to study the response of HBE cells to different substrate stiffness. The migration speed maps (Figure 3.5 (b)) show that cells are more migratory when

culturing on AAm/BIS 20% gel than cells cultured on AAm/BIS 10% gel and Transwell membrane (control group) after three weeks of ALI culture.

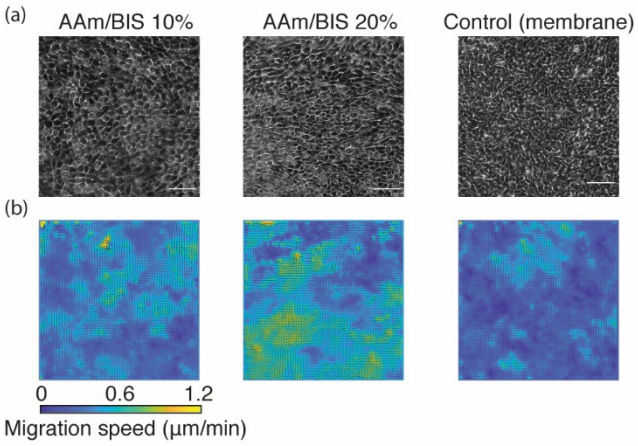



**Figure 3.5** (a) Confocal microscope images show HBE cell morphology under different substrate stiffness for ALI culture at Day 21. (b) The speed maps show cell migration under different substrate stiffness on ALI culture day 7, 14 and 21. Scale bar: 100 μm.

We quantify cell migration speed and discover that HBE cells on PAAm AAm/BIS 10% gel are more migratory compared to the control on ALI day 21, and cells on PAAm AAm/BIS 20% gel migrate far faster than cells on AAm/BIS 10% gel. On ALI day 14, HBE cells on PAAm AAm/BIS 20% gel still migrate faster than those on AAm/BIS 10% gel, while there is no significant difference between cells on PAAm AAm/BIS 10% gel and control. On ALI day 7, cell migration shows no difference among the three groups.

Furthermore, the cell migration speed on PAAm gel and Transwell membrane all increase on ALI day 14, especially for the cells on AAm/BIS 20% gel where the migration speed rises significantly (green bars in Figure 3.6). Although the increased migration speeds might be attributed to slower proliferation or weaker cell adhesion, the migration speed manage to decrease after three weeks of ALI culture. Moreover, the migration of epithelial cells correlates with cell

density [29]. When a confluent monolayer of cells reaches high cell density, cells are more static and less migratory. As previously shown, HBE cells culturing on different substrate stiffness have significantly different cell densities after weeks of ALI culture, corresponding to different cell migration speeds. As a result, HBE cells culturing on the Transwell membrane show the highest cell density and lowest cell migration speed. Yet, cells culturing on AAm/BIS 20% gel show extremely high migration speed which does not correspond to the cell density, indicating the effect of substrate stiffness on HBE cell migration. These results raise the question of whether substrate stiffness affects cell mobility. Next, we study cell mobility through the use of cell shape index.

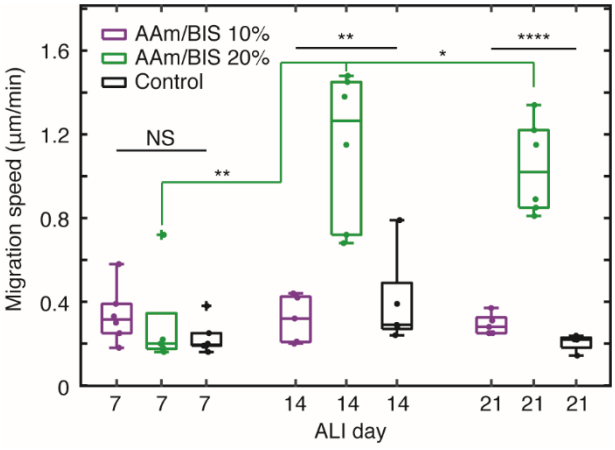



**Figure 3.6** The cell migration speeds of HBE cells under different substrate stiffness on ALI culture day 7, 14 and 21. * $p < 0.1$, ** $p < 0.01$, **** $p < 0.0001$. Data is analyzed by one-way ANOVA.

### 3.5 Cell shape index of HBE cells in PAAm gel-ALI system

The difference in cell migration can be further interpreted with unjammed-to-jammed transition [30]. When the cells are in an unjammed state, meaning the cells can move through the tissues and have high mobility. Otherwise, the cells are packed tightly and the shape may transition from unjammed state to a jammed state. The unjammed-to-jammed transition can be determined by quantifying an index called cell shape index. Cell shape index, defined as cell perimeter divided by the square root of cell area [30], correlates to cell shape and structural stability. A high cell shape index occurs when the cell is immature and unstable due to its irregular, elongated cell shape. When the cell shape index decreases and approaches 3.81, the transition occurs and the cells become static and jammed.

To measure cell shape index, we obtain bright field images using phase-contrast mode on ALI day 7, 14, and 21. We randomly choose 4 images per well and 3 wells in total. We randomly select 25 cells in each well, resulting in collecting 100 cells per well. We use Fiji software to quantify cell area and cell parameter, and then employ cell shape index to quantify cell morphology.

Quantitatively, cells on AAm/BIS 10% hydrogel have a larger cell shape index with a median value of 4.2 on ALI day 7 (Figure 3.7) where the cell shape index of cells on AAm/BIS 20% gel is 4, indicating soft substrate stiffness might affect cell shape and stability. After three weeks of ALI culture, the cell shape index shows no significant difference between various substrate stiffness which implies the maturity of HBE cells. Moreover, the cell shape index decreases from ALI day 7 to day 21, which verify that mature cells have round or symmetric shape. These results demonstrate that substrate stiffness affects the structure of HBE cells under ALI culture.

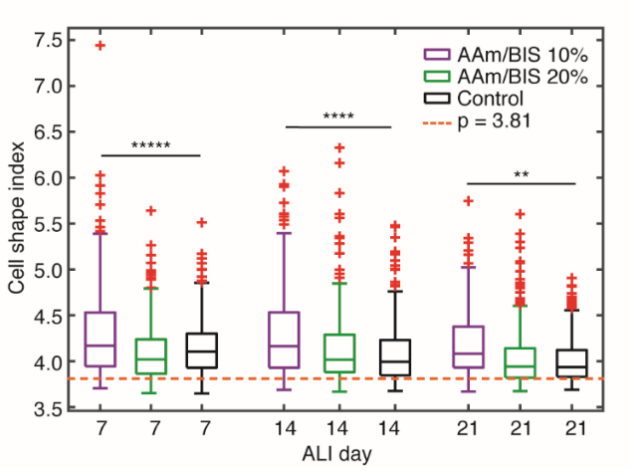



**Figure 3.7** Cell shape index of HBE cells under different substrate stiffness on ALI culture day 7, 14 and 21. ** $p < 0.01$, **** $p < 0.0001$, ***** $p < 0.00001$. Data is analyzed by one-way ANOVA. Each group contains 300 data points.

### 3.6 Summary and future work

In summary, we have developed a gel-coated air-liquid-interface culture system to mimic the mechano-environment of airway epithelial cells *in vitro*. Polyacrylamide hydrogel is used as the ECM mimicking matrix. The stiffness of polyacrylamide hydrogel can be tuned by controlling the acrylamide and bis-acrylamide concentration to match the stiffness of healthy and diseased lung tissues. We show that the gel-ALI culture system allows biomolecules in cell culture media to transport through and provide nutrients to the HBE cells. In the gel-ALI culture system, HBE cells can proliferate to reach confluency and further differentiate to ciliated cells and goblet cells, forming a pseudostratified epithelium. Use gel-ALI system, we discover that the cell shape, cell density and cell migration are correlated with the substrate stiffness. The gel-ALI culture system provides a useful model to study human airway biology and diseases.

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# Appendix

## Materials and Chemicals

Ammonium persulfate (APS, ≥98%), acrylamide (AAm, 40%), N, N′-methylene bis-acrylamide (BIS, 2%) and tetramethylethylenediamide (TEMED, ~99%) were brought from Simga-Aldrich and used as arrived. Sulfo-SANPAH was purchased from Fisher Scientific. Fluorescent labeled dextran (Texas Red™, MW 70,000 Da) was purchased from Fisher Scientific. Type I collagen solution (PureCol®, 3mg/ml) was bought from Advanced Biomatrix. PneumaCult™-ALI Medium and PneumaCult™-Ex Plus Basal Medium were purchased from STEMCELL Technologies. Dulbecco's phosphate buffered saline (DPBS, without calcium) was purchased from Fisher Scientific. Paraformaldehyde (purified, Spectrum™), bovine serum albumin (BSA, heat shock treated), 4',6-Diamidino-2-Phenylindole (DAPI), and dithiothreitol (DTT) were brought from Fisher Scientific. Triton X-100 detergent was purchased from Simga-Aldrich. Transwell® permeable supports (12mm insert, 12 well plate, 0.4μm polyester membrane, tissue culture treated) were purchased from Corning.

## Permeability of different thickness PAAm gel in gel-coated ALI system

The permeability of PAAm gel on Transwell inserts is further investigated by the variation in hydrogel stiffness. Normalized diffusion profiles are computed and are shown in Figure S1(a) for AAm/BIS 10% gel and Figure S1(b) for AAm/BIS 20% gel. Comparing the intensity change AAm/BIS 10% gel (Figure S1(a)) at x = 0 in each time interval to the total intensity change of one hour, it suggests that most of the dextran particles diffuse rapidly in the first 20 minutes for 100 μm gel, while it might need to take 40 minutes for most of the dextran to diffuse through the 250 μm gel. As for AAm/BIS 20% gel, the diffusion of dextran is smaller compared to the AAm/BIS

10% gel (Figure S1(b)). Moreover, the difference in the intensity change in the first 20 minutes is less significant in AAm/BIS 10% gel. This is likely because AAm/BIS 20% gel has a smaller mesh size, and the dextran particles are easier to be trapped in the hydrogel network. Yet, the intensity remains continuously increasing during the first one hour. Collectively, our results demonstrate that large metabolic biomolecules in ALI culture media can diffuse through the PAAm hydrogel layer and reach the cells on the gel surface.

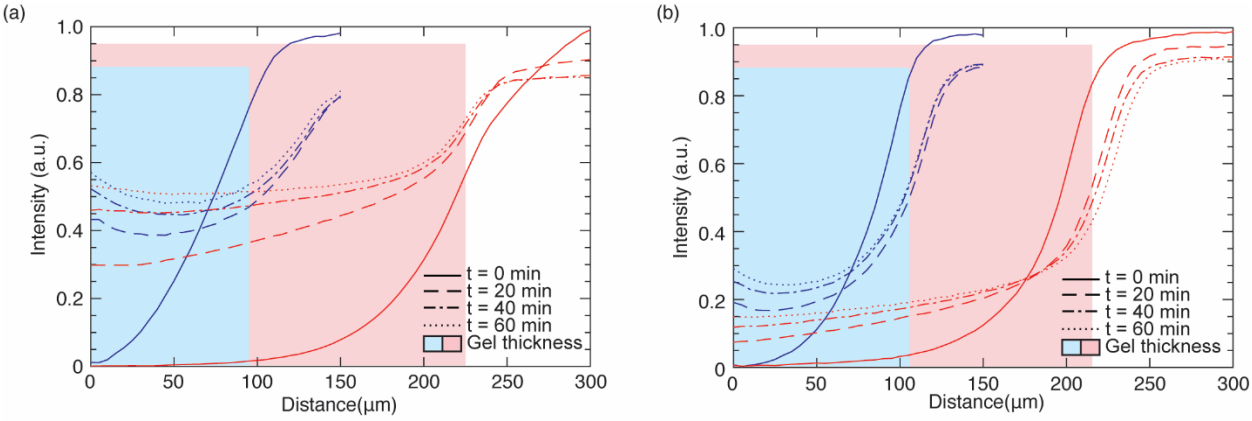


**Figure S1** (a) Dependence of fluorescence intensity on distance from the AAm/BIS 10% gel above the Transwell membrane to the apical chamber under different gel thickness. (b) Dependence of fluorescence intensity on distance from the AAm/BIS 20% gel above the Transwell membrane to the apical chamber under different gel thickness.

**Cell area of HBE cells in PAAm gel-ALI system**

To examine the morphological changes of HBE cells under different substrate stiffness, we quantify the cell area on ALI day 7, 14, and 21 (Figure S2). Quantitatively, cells growing on PAAm hydrogel have a larger cell area (400 $\mu m^2$/cell on AAm/BIS 20% gel) compared to cells on the Transwell membrane on ALI day 21 (210 $\mu m^2$/cell). For HBE cells in the gel-ALI system, cells on AAm/BIS 10% gel have a slightly larger cell area with a median value of 430 $\mu m^2$/cell. These

results indicate substrate stiffness is associated with cell area and cell growth. Yet, the increased cell area on soft substrate slows down cell proliferation. Nevertheless, the cells on PAAm hydrogel have the capacity to differentiate into ciliated cells and goblet cells.

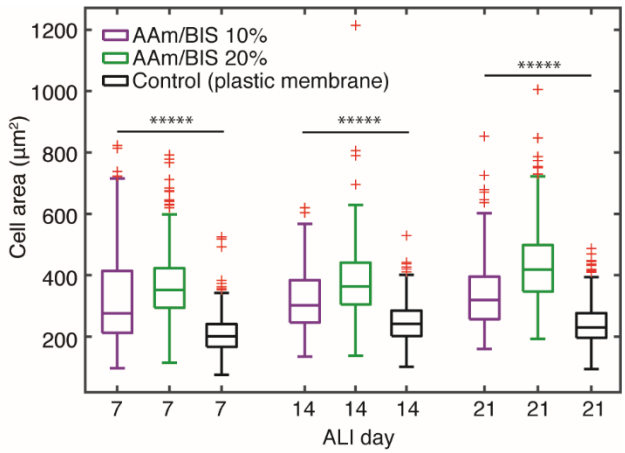


**Figure S2** The average cell area of HBE cells under different substrate stiffness on ALI culture day 7, 14 and 21. ***** $p < 0.00001$. Data is analyzed by one-way analysis of variance (ANOVA).