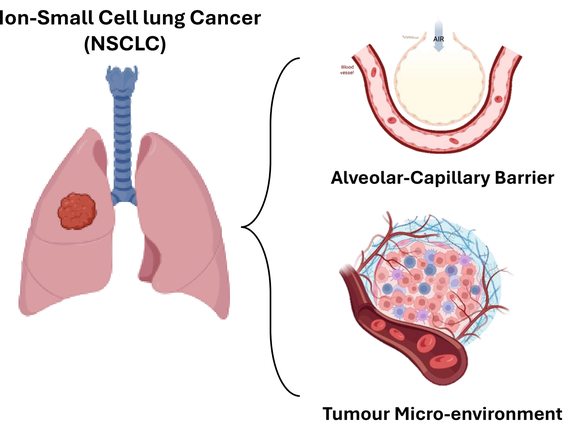
Biomimetic Agarose–Gelatin Membranes for 3D Lung Cancer Models under Air–Liquid Interface Conditions

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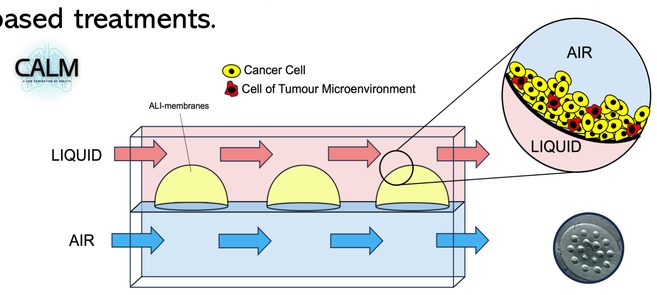
BACKGROUND

The development of **advanced in vitro models** is essential to better reproduce the structural and microenvironmental complexity of tumors. Conventional **two-dimensional systems fail to capture key aspects** such as cell–cell interactions, spatial organization, and physiologically relevant gradients.
In the context of **lung cancer**, there is a growing need for models compatible with **aerosol-based drug delivery**, which represents a promising therapeutic strategy. **Air–liquid interface** (ALI) culture conditions provide a more physiologically relevant environment by exposing the epithelial surface to air while ensuring nutrient supply from the basal compartment.



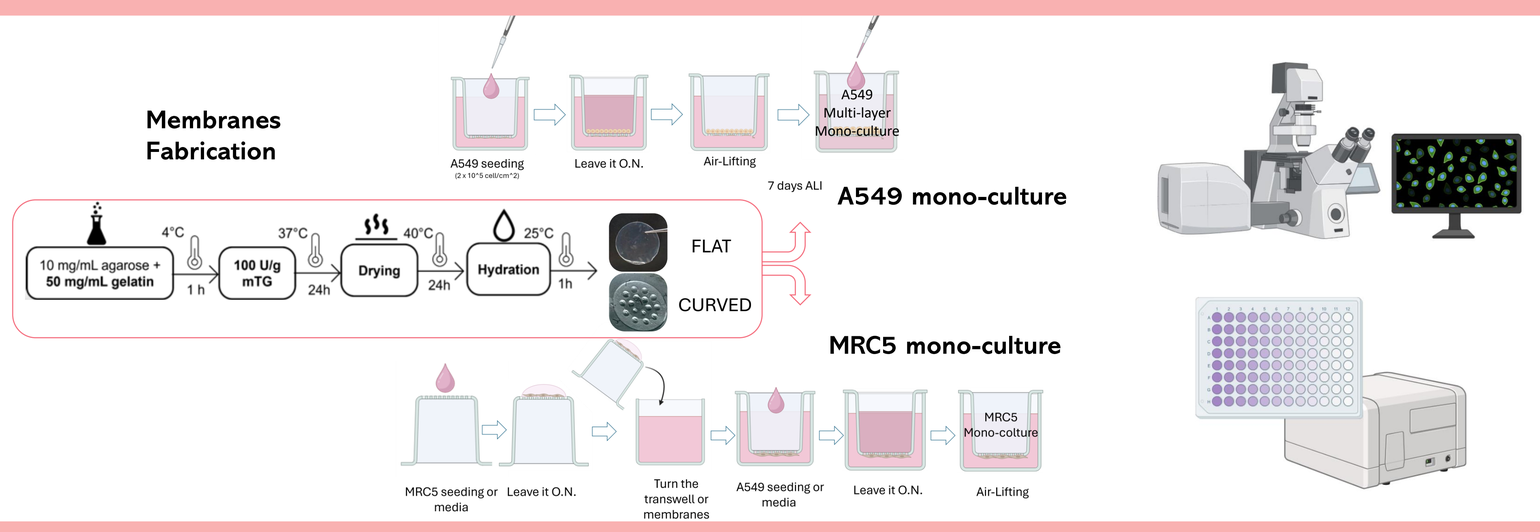
AIM

This study aims to **develop biomimetic agarose–gelatin interpenetrating network membranes crosslinked with microbial transglutaminase** (Ag/Gel+mTG) as substrates for engineering three-dimensional lung cancer models. The goal is to establish a **versatile platform capable of supporting both epithelial multilayer cultures and tumor–stroma co-culture systems**, while remaining compatible with aerosol-based treatments.



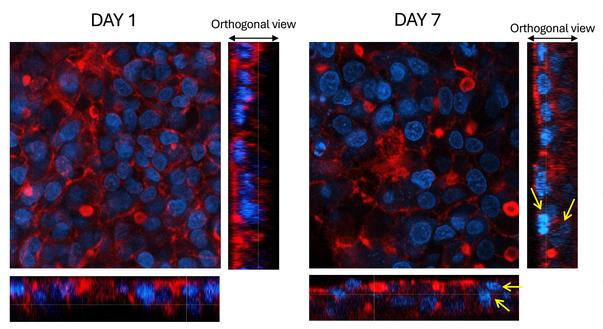
MATERIALS and METHODS

- Agarose–gelatin membranes were **enzymatically crosslinked with microbial transglutaminase**, enabling stable flat and curved constructs (~600 µm) mounted on CellCrown inserts
- Under ALI conditions, **A549 cells** formed the apical epithelial layer, while **MRC-5 fibroblasts** established the basolateral stromal compartment
- Tissue organization and EMT were analyzed via **confocal immunofluorescence** of vimentin and E-cadherin, with fibroblast viability assessed using **CellTiter-Glo** (CTG) assay

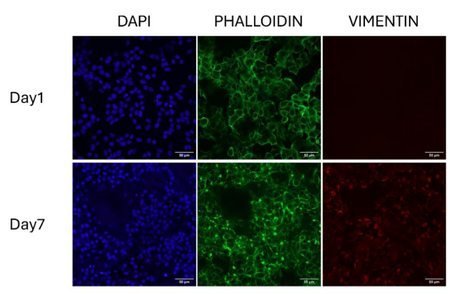


MATERIALS and METHODS

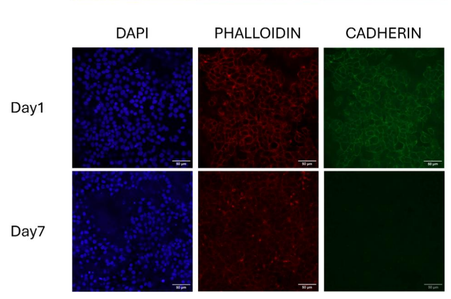
- Under ALI conditions, **A549 cells progressively organized into stratified epithelial structures**, forming reproducible and well-defined multilayer assemblies within 7 days, with enhanced vertical growth compared to conventional 2D cultures.
- Confocal immunofluorescence analysis showed **EMT-associated phenotypic changes,** with increased expression of vimentin and reduced E-cadherin, suggesting a shift toward a more mesenchymal and potentially aggressive tumor phenotype.
- MRC-5 fibroblasts cultured on the basolateral side **maintained** their characteristic **morphology and viability on Ag/Gel+mTG membranes**, as confirmed by CellTiter-Glo (CTG) assay, supporting long-term culture and multicellular tumor–stroma model development.



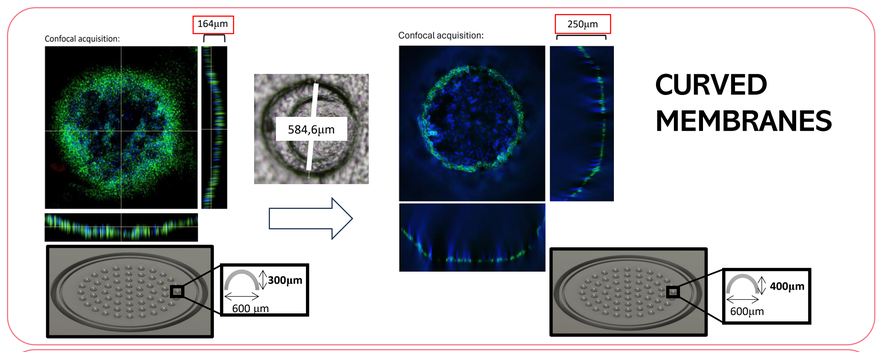
Z-stacks at confocal microscope (Nikon, model V2V3, system NKN-L5M5-D9X2). A549 cells were stained with Phalloidin 647 (red) and DAPI (blue). Stacks were collected using 20X magnification with air objective. Day1 stack is 44 microns, Day7 stack is 130 microns.

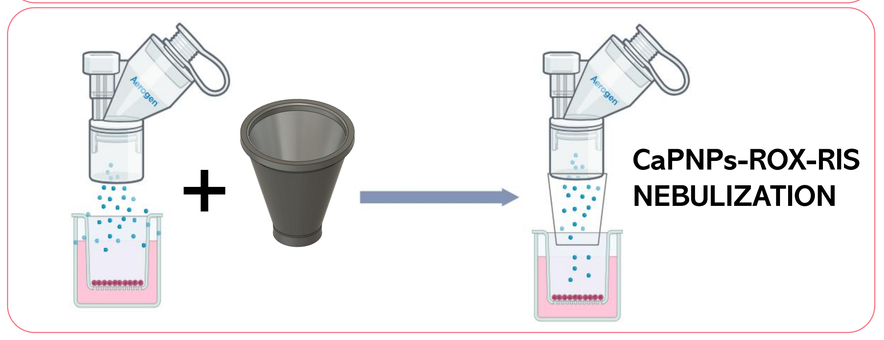


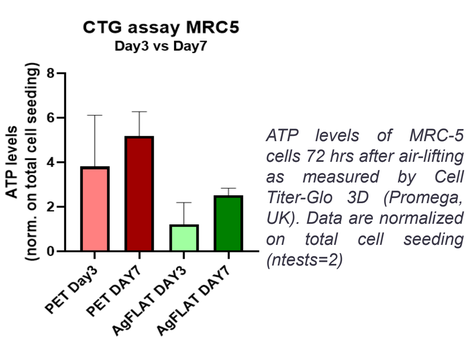
Confocal microscope acquisition (Nikon, model V2V3, system NKN-L5M5-D9X2). A549 cells were stained with Phalloidin 488 (green), DAPI (blue) and vimentin primary antibody with 647 Alexa fluor (red) secondary antibody. Images were collected using 20X magnification with air objective.



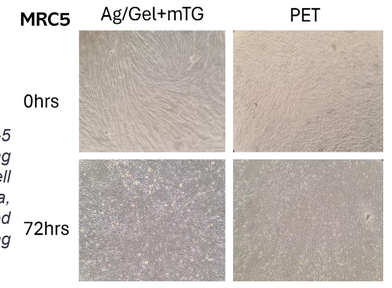
Confocal microscope acquisition (Nikon, model V2V3, system NKN-L5M5-D9X2). A549 cells were stained with Phalloidin 647 (red), DAPI (blue) and cadherin primary antibody with 488 Alexa fluor (green) secondary antibody. Images were collected using 20X magnification with air objective.







ATP levels of MRC-5 cells 72 hrs after air-lifting as measured by Cell Titer-Glo 3D (Promega, UK). Data are normalized on total cell seeding (ntests=2)



Acquisitions at optical microscope using 10X magnification of MRC-5 cells grown on the basolateral side of on Ag/Gel + mTG membranes, soon after air-lifting day (0 hrs) and 72 hrs after air-lifting.

RESULTS

The developed membranes effectively support the formation of **structurally complex 3D lung cancer models under physiologically relevant ALI conditions**. The observed EMT-associated changes highlight the **influence of the 3D microenvironment** on cell phenotype.
Importantly, the co-culture configuration enables the investigation of heterotypic interactions, which play a critical role in cancer progression and therapeutic response. Compared to traditional in vitro systems, this platform **provides improved biological relevance and experimental flexibility**.
Ag/Gel+mTG membranes represent a versatile platform for engineering three-dimensional lung cancer models. Their compatibility with ALI culture and aerosol exposure makes them particularly suitable for studying respiratory tumors and evaluating efficient inhaled therapies.
This system probably could fill the gap between simplified in vitro models and more complex in vivo conditions, offering a valuable tool for preclinical research.
Ongoing work focuses on integrating immune components into the model to further enhance its physiological relevance. In addition, future studies will evaluate the response of these engineered tissues to nebulized chemotherapeutic agents, with the aim of improving preclinical assessment of aerosol-based cancer therapies.